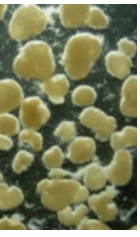
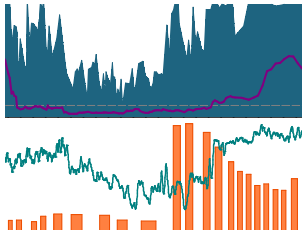
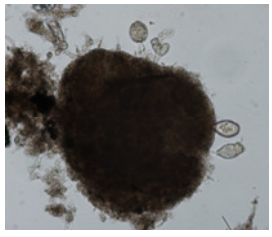
Biological nutrient removal in SBR technology: from floccular to granular sludge

Marta Coma Bech

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PhD THESIS

BIOLOGICAL NUTRIENT REMOVAL IN SBR TECHNOLOGY: FROM FLOCCULAR TO GRANULAR SLUDGE

MARTA COMA BECH

2011

Dirigida per:
Dr. Jesús Colprim Galceran
Dr. Sebastià Puig Broch

PROGRAMA DE DOCTORAT DE CIÈNCIES EXPERIMENTALS I SOSTENIBILITAT

Universitat de Girona

El Dr. JESÚS COLPRIM GALCERAN i el DR. SEBASTIÀ PUIG BROCH,
Professors del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona,

CERTIFIQUEN

Que aquest treball, titulat "**Biological nutrient removal in SBR technology: from floccular to granular sludge**", que presenta la llicenciada MARTA COMA BECH per a l'obtenció del títol de doctora, ha estat realitzat sota la seva direcció i que compleix els requeriments per poder optar a Menció Europea.

I perquè en prengueu coneixement i tingui els efectes que correspongui, presentem davant la Facultat de Ciències de la Universitat de Girona l'esmentada Tesi, signant aquesta certificació a

Girona, 6 de Maig del 2011

Jesús Colprim Galceran Sebastià Puig Broch

A totes les dones que m'han fet costat,
en especial a la Marilós.

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."
Sir William Bragg

"Aim for success, not perfection. Never give up your right to be wrong, because then you will lose the ability to learn new things and move forward with your life."
Dr. David M. Burns

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El següent apartat és l'única part d'una tesi que tothom, sense excepció, es llegeix (si ets un d'ells, no et sentis culpable, no ets l'únic). Això, però, fa que la tasca d'escriure'ls sigui més difícil, perquè un doctorat (gairebé etern) s'acaba gràcies a la contribució de moltes persones. Per aquesta raó, seguint un bon consell, i sense que serveixi de precedent, enumeraré els agraïments tal i com es fa en un article científic, ordenant els autors segons la contribució en el treball. *Enjoy it*!

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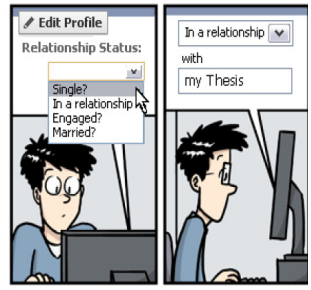

Gina, les paraules sobren. Gràcies per obrir-me les portes de casa teva quan més ho necessito, ara i sempre!

Comics from "Piled Higher and Deeper" by Jorge Cham © www.phdcomics.com
"How do I love you?" 02/11/2011; "And after that?" 10/23/2009; "Relationship status" 01/21/2011

Les meves nenes: Ariadna, Gemma, Marta, Clàudia, Nuri, Ju, Txell i Mallen, per compartir molts moments, per trobar estones per xerrar seriosament i altres per riure sense parar i poder oblidar aquelles situacions que ens fan trontollar.

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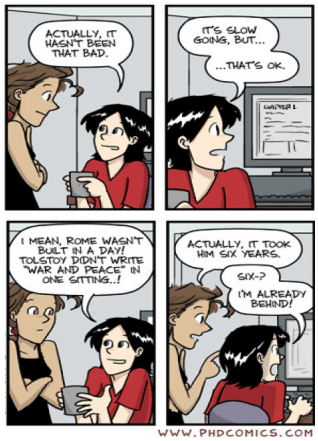

Ha arribat el torn de l'extensa llista de lequians els quals cal incloure les *viejas glorias* (Helio, Lluís, Anna, Sergio, Dalmau, ...), la meva *quinta* (Carla, Alba, Esther, Hèctor, Maël i Pau), les noves incorporacions (Jordi, Tico, Xavi, Laura, Marc, Marina, Sara, Montse, Jordi, ...), les *jefas* del labo (Ariadna, Anna) i la gran quantitat de gent que ha passat pel grup (Marta, Joan, Anna, Davide, Natàlia, Íngrid ...) els quals tots heu deixat alguna petjada en el camí seguit. Moltes gràcies!

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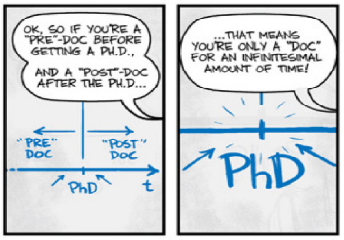
"Red ink" 03/07/2006; "Extraneous" 07/28/2010; "Denial" 08/02/2010; "Write in pieces" 08/04/2010



Toca el torn de la penya de l'espardenya: Anna Dachs, Anna Díaz, Santi, Naiara, Ferran, Xavi, Alba, Maira, Mònica F., Mònica B. i Quim. La meitat de nosaltres es pregunta perquè això del doctorat i l'altre meitat encara no entenen què fem... Espero que encara ens trobem anualment per discutir sobre el tema!

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M.Coma. Girona, 20 febrer 2011

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"Instantaneous property" 08/20/2010; "Paste together" 04/24/2009; "Denial" 08/02/2010; "Is this even English?" 05/18/2007

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A or ANA	Anaerobic phase
AE or AER	Aerobic phase
AGS	Aerobic Granular Sludge
AnGS	ANaerobic Granular Sludge
Anammox	Anaerobic AMMonium Oxidation
AO or ANX	Anoxic phase
AOB	Ammonium Oxidizing Bacteria
ATP	Adenosine Triphosphate
A^2O	Anaerobic-Anoxic-Aerobic configuration
AV	Ammonia Valley
BOD_5	Biochemical Oxygen Demand after 5 days [mg BOD·L^{-1}]
BNR	Biological Nutrient Removal
C	Carbon [mg C·L^{-1}]
CLSM	Confocal Laser Scanning Microscope
CO_3^{2-}	Carbonate [mg C·L^{-1}]
$COD_{T\ or\ S}$	Chemical Oxygen Demand (Total or Soluble) [mg COD·L^{-1}]
COD_T /N	Carbon/Nitrogen ratio [g COD·g^{-1} N]
COD_T /N/P	Carbon/Nitrogen/Phosphorus ratio [100 g COD: X g N_T: X g P]
COD_T/P	Carbon/Phosphorus ratio [g COD·g^{-1} N]
DME	Dehydrated Meat Extract
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon [mg C·L^{-1}]
DPAO	Denitrifying Phosphate Accumulating Organism
EBPR	Enhanced Biological Phosphorus Removal
EC	European Community
EtOH	Ethanol
EUB	Eubacteria
EWFD	European Water Framework Directive
FA	Free Ammonia [mg N-NH_3·L^{-1}]
FISH	Fluorescent *In Situ* Hybridization
FNA	Free Nitrous Acid [mg N-HNO_2·L^{-1}]
GAO	Glycogen Accumulating Organisms

GC	Gas Chromatography
H_2CO_3	Carbonic acid [mg C·L^{-1}]
HCO_3^-	Bicarbonate [mg C·L^{-1}]
HD	Heptadecane
HRT	Hydraulic Retention Time [days]
IC	Inorganic Carbon [mg C·L^{-1}]
LEQUIA	Laboratory of Chemical and Environmental Engineering (*Laboratori d'Enginyeria Química i Ambiental*)
$minSRT_{AER}$	Minimum Aerobic Sludge Residence Time [days]
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
N	Nitrogen [mg N·L^{-1}]
NA	Nitrate Apex
NLR	Nitrogen Loading Rate [g N·$m^{-3}d^{-1}$]
$N\text{-}NH_4^+$	Ammonium [mg $N\text{-}NH_4^+$·L^{-1}]
$N\text{-}NO_2^-$	Nitrite [mg $N\text{-}NO_2^-$·L^{-1}]
$N\text{-}NO_3^-$	Nitrate [mg $N\text{-}NO_3^-$·L^{-1}]
NOB	Nitrite Oxidizing Bacteria
Norg	Organic Nitrogen [mg N·L^{-1}]
N-TN	Total Nitrogen [mg N-TN·L^{-1}]
N-TKN	Total Kjeldahl Nitrogen [mg N-TKN·L^{-1}]
OLR	Organic Loading Rate [Kg COD·$m^{-3}d^{-1}$]
OM	Organic Matter [mg COD·L^{-1}]
ORP	Oxidation Reduction Potential [mV]
P	Phosphorus [mg P·L^{-1}]
PAO	Phosphorus Accumulating Organisms
PHA	Poly-β-HydroxyAlkanoates
PHB	Poly-β-HydroxyButirate
PHV	Poly-β-HydroxyValerate
PH2MV	Poly-β-Hydroxy-2-MethylValerate
PLR	Phosphorus Loading Rate [g P·$m^{-3}d^{-1}$]
PolyP	Polyphosphate
$P\text{-}PO_4^{3-}$	Phosphate [mg $P\text{-}PO_4^{3-}$·L^{-1}]
P-TP	Total phosphorus [mg P-TP·L^{-1}]
PUR	Phosphate Uptake Rate [mg $P\text{-}PO_4^{3-}$·$g^{-1}VSSh^{-1}$]

RO	Reverse Osmosis
rRNA	Ribosomic RiboNucleic Acid
SBR	Sequencing Batch Reactor
SND	Simultaneous Nitrification and Denitrification
SNDPR	Simultaneous Nitrification-Denitrification and Phosphorus Removal
SRT	Sludge Residence Time [days]
SRT_{AER}	Aerobic Sludge Residence Time [days]
SVI	Sludge Volumetric Index [$mL \cdot g^{-1}$ TSS]
SVI_{10}	Sludge Volumetric Index after 10 minutes [$mL \cdot g^{-1}$ TSS]
SVI_{30}	Sludge Volumetric Index after 30 minutes [$mL \cdot g^{-1}$ TSS]
T	Temperature [ºC]
TCA	TriCarboxilic Acid
TOC	Total Organic Carbon [mg $C \cdot L^{-1}$]
TSS	Total Suspended Solids [mg or g $TSS \cdot L^{-1}$]
UCT	University of Cape Town
UdG	University of Girona (*Universitat de Girona*)
UNL	Universidade Nova de Lisboa
VER	Volume Exchange Ratio [%]
VFA	Volatile Fatty Acids [mg $C \cdot L^{-1}$]
VIP	Virginia Initiative Plant
V_{max}	Maximum reactor volume [L]
V_{min}	Minimum reactor volume [L]
VSS	Volatile Suspended Solids [mg or g $VSS \cdot L^{-1}$]
V_{30}	Settled sludge volume after 30 minutes [$mL \cdot L^{-1}$]
WW	Wastewater
WWTP	WasteWater Treatment Plant


SUMMARY

Summary

Nutrient removal is a key factor in maintaining the natural water cycle following human interference, and continued population growth has made domestic wastewater treatment both a requirement and a challenge in all around the world. Higher flows have to be handled in wastewater treatment plants (WWTPs) built years ago or in newly designed plants with reduced surface areas. Sequencing batch reactors (SBRs) have been studied and used for years because of their relatively small footprint. They can be easily adapted to regulatory changes in effluent parameters such as nutrient removal, but even more compact systems than SBRs are required to meet land limitations and high nutrient loads.

Biological nutrient removal (BNR) has been studied and applied for decades. However, more anthropogenic uses and the continued demand for water have forced WWTPs to operate at their maximum capacity. An increase in load can often lead to destabilization of the microbial population in the system and, as a consequence, the breakdown of the nutrient removal process. When the disturbance from these reactions occurs in the same basin, the stability of the system is sometimes hard to manage because different conditions are required to perform nutrient (nitrogen and phosphorus) removal. Working with both processes simultaneously demands a compromise in operating conditions. Hence, when a destabilization occurs, consecutive steps focusing individually on each nutrient have to be carried out before coupling together all the reactions. Against this background, this thesis describes a practical guide to recover and stabilize nitrogen and phosphorus removal after a load increase. It focuses on operational strategies such as feed pattern and phase length distribution. Nitrification has to be the first process to be recovered as it is severely affected by biomass washout and low sludge age, consequences of an increase in the volume of wastewater. Increasing the aerobic phase at the expense of part of the anaerobic phase improved nitrification, but even more improvement was achieved by applying longer aerobic periods and avoiding transient responses between phases. When ammonium oxidation increased, nitrate concentration in the reactor increased as well. The efficiency of denitrification was the next goal, as nitrate was the main compound interfering

with phosphorus removal. When working with a step-feed strategy, a higher volume distribution at the beginning of the cycle reduced the amount of nitrate in the effluent. At the same time, more organic matter was available for enhanced biological phosphorus removal (EBPR) performance. Finally, sufficiently long anaerobic phases needed to be applied to improve phosphorus removal, the last goal of the stabilization strategy.

Because of its compactness and reduced production of sludge, in recent years aerobic granular sludge has been proposed as an alternative for wastewater treatment. Compared to conventional activated sludge, these self-immobilized microorganisms allow high amounts of active biomass with a dense, strong microbial structure and good settleability to accumulate in the reactor. All these characteristics make aerobic granules a reliable alternative to reduce space and increase loading rates in treatment plants. However, there are still many problems to be solved in the granulation process. Furthermore, when granules develop with low-strength wastewater such as domestic wastewater, the low organic loading rate (OLR; less than 1 Kg $COD \cdot m^{-3} d^{-1}$) results in slower formation and a longer time to reach a steady state.

In this thesis comparative studies to investigate granulation have been carried out with synthetic and real wastewater. First, a settling time reduction technique was applied to select faster biomass aggregates over slower settling flocs. This method was tested in identical reactors treating different volumes of wastewater. It was observed that volume exchange ratios of 40-50% enhanced granule formation more than higher values, even though they increased the loading rate of the system. This was mainly due to a higher washout of biomass, affecting both granulation and nutrient removal performance. When dealing with real wastewater, one of the main problems is the small fraction of soluble organic matter. When focusing on granular reactors, raw wastewater favored the aggregation of floccular biomass more than settled or decanted wastewater, as particles from the influent provided higher organic loading rates.

When granular sludge was used for BNR, microorganisms were placed in the same biomass aggregate instead of in different reactor basins. As a result, the

nitrogen and phosphorus bioreactions interacted more strongly. Simultaneous nitrification and denitrification, sometimes together with phosphorus removal, was observed when low-strength wastewater was being treated because oxygen diffused into the granules. The influence of nitrite and nitrate, as products of nitrification, on simultaneous processes in granular biomass was evaluated. Both nitrite and nitrate were used for simultaneous nitrogen and phosphorus removal, although the same quantity of nitrate removed more phosphorus than nitrite did. In contrast, nitrification-denitrification via nitrite pathway would reduce the requirement for organic matter. However, the nitrite concentration must be taken into account as phosphorus removal could be inhibited when large amounts of free nitrous acid are produced. Nitrite enhanced simultaneous removal when the granules were big enough to diffuse nutrients into the core and prevented inhibition processes.

Resum

L'eliminació de nutrients és una de les majors preocupacions per a mantenir el cicle natural de l'aigua després de la influència de l'home. El creixement continuat de la població en totes les parts del planeta fa del tractament de les aigües residuals un requeriment i un desafiament al mateix temps. Les estacions depuradores d'aigües residuals (EDAR*;* acrònim en anglès, *WWTP*) han de tractar cabals més elevats amb les mateixes instal·lacions construïdes anys enrere. La necessitat de construir noves EDAR en àrees més reduïdes és un altre problema a resoldre. Amb aquesta última finalitat, els reactors discontinus seqüencials (RDS; acrònim en anglès, *SBR*) han estat el punt de mira de diferents estudis i aplicacions gràcies a la seva reduïda empremta pel que fa l'espai. A més a més, aquests reactors són extremadament flexibles i adaptables a canvis d'operació per a la regulació de l'efluent, com seria l'eliminació de nutrients. Tot i això, la problemàtica de les limitacions en espai i el tractament d'altes càrregues desemboca en la recerca de sistemes més compactes.

L'eliminació biològica de nutrients (EBN; acrònim en anglès*, BNR*) ha estat investigada durant dècades. Per altra banda, la continua demanda d'aigua força les EDAR a treballar a la seva màxima capacitat. L'increment de càrrega comporta eventualment una desestabilització de la població microbiana i, com a conseqüència, la pèrdua del procés d'eliminació de nutrients. Quan la pertorbació de les reaccions biològiques es produeix en un mateix reactor, la recuperació de l'estabilitat del sistema es troba lligada a les diferents condicions d'operació requerides per a l'eliminació de nutrients com el nitrogen i el fòsfor. Treballar amb ambdós processos de manera simultània requereix una solució de compromís pel que fa a les condicions d'operació. Per aquesta raó, s'han de dur a terme diferents passos consecutius per a cada nutrient quan el sistema es desestabilitza abans d'acoblar totes les reaccions biològiques. Amb aquesta finalitat, la present tesi descriu un protocol de treball per a la recuperació i estabilització de l'eliminació biològica de nitrogen i fòsfor després d'un increment de càrrega. L'estudi es centra en les diferents condicions d'operació com les estratègies d'alimentació o la distribució de

durada de fases. La nitrificació hauria de ser el primer procés a recuperar ja que es veu fortament afectat per una baixa edat del fang, possible conseqüència de l'augment d'aigua a tractar. Allargar la fase aeròbia a compte d'una part de la fase anaeròbia, però encara més aplicar llargs períodes aerobis evitant els temps de resposta entre fases, milloraria el procés de nitrificació. Quan l'oxidació d'amoni recupera, la concentració de nitrats creix en el si del reactor. L'eficiència de desnitrificació, doncs, haurà de ser el pròxim objectiu, ja que els nitrats són l'interferent principal de l'eliminació de fòsfor a causa de la competència dels dos processos per la matèria orgànica. Quan es treballa amb una estratègia d'alimentació per etapes, una distribució de volum major a la primera alimentació a l'inici del cicle reduirà la quantitat de nitrats a la sortida del reactor. Al mateix temps, més matèria orgànica restarà disponible per al procés d'eliminació biològica de fòsfor. Finalment, com a últim pas, les fases anaeròbies hauran de ser prou llargues per a estabilitzar la captació de matèria orgànica i augmentar la bioacumulació de fòsfor.

Fent referència a la compactació i la reducció de la producció de biomassa activa, el fang granular aerobi ha estat proposat durant els últims anys com a alternativa per al tractament d'aigües residuals. Comparat amb els sistemes convencionals de fangs actius, aquests microorganismes encapsulats permeten l'acumulació de grans quantitats de biomassa activa en un sol reactor, presentant una estructura microbiològica densa i una bona sedimentabilitat. Totes aquestes característiques permeten que el fang granular aerobi es proposi com una alternativa viable per a reduir l'espai de les depuradores i tractar càrregues més elevades. Per contra, el procés de granulació té encara molts buits de coneixement que cal investigar. A més a més, quan es desenvolupen sistemes granulars amb aigües residuals de baixa càrrega, com les aigües urbanes les quals presenten càrregues de matèria orgànica menors a 1 Kg DQO·$m^{-3}d^{-1}$, el procés de formació s'alenteix i són necessaris períodes més llargs per assolir l'estat estacionari.

En la present tesi s'han realitzat estudis comparatius de granulació amb aigua residual sintètica i real amb concentracions similars a l'aigua urbana. Primerament es va aplicar una estratègia per a reduir el temps de sedimentació amb l'objectiu de seleccionar els agregats de biomassa amb sedimentació més

ràpida. Aquesta metodologia es va dur a terme en reactors idèntics, tractant diferents volums d'aigua residual. Es va observar que relacions volumètriques de canvi de 40-50% milloraven la formació de gràNul, en contrast amb relacions de canvi majors, malgrat aquestes últimes incrementaven la càrrega aplicada sobre el sistema. Aquest fet era degut principalment a un major rentat de la biomassa, afectant tant el procés de granulació com el rendiment d'eliminació de nutrients. Un dels principals problemes pel tractament d'aigües residuals urbanes és el baix contingut de matèria orgànica soluble. Pel que fa a reactors granulars, la utilització d'aigua residual sense pre-tractar en comptes d'aigua residual sedimentada o decantada pot afavorir l'agregació de biomassa flocular, perquè l'afluent aporta càrregues orgàniques més elevades.

Pel que fa a l'EBN en fang granular, els microorganismes es situen en el mateix agregat de biomassa en comptes de diferents reactors. Per aquesta raó, les bioreaccions del nitrogen i el fòsfor tenen unes interaccions més fortes entre elles. El procés simultani de nitrificació i desnitrificació, a vegades junt amb l'eliminació biològica de fòsfor, s'ha observat pel tractament d'aigües residuals de baixa càrrega degut a la difusió d'oxigen en els gràNuls. La influència del nitrit i el nitrat com a productes de la nitrificació es va avaluar en processos simultanis en fang granular. Tant nitrit com nitrat van ser utilitzats per a l'eliminació simultània de nitrogen i fòsfor, tot i que a igual concentració d'ambdós, el nitrat permetia una major eliminació de fòsfor. Contràriament, la nitrificació-denitrificació via nitrit permetria reduir els requeriments de matèria orgànica. No obstant això, cal tenir en compte la concentració de nitrit perquè l'eliminació de fòsfor pot veure's inhibida en presència d'elevades concentracions d'àcid nitrós. Així doncs, el nitrit pot millorar l'eliminació simultània en gràNuls si les partícules presenten una mida suficientment gran que permeti la difusió dels nutrients al centre i previngui els processos d'inhibició.

Resumen

La eliminación de nutrientes es una de las mayores preocupaciones para mantener el ciclo natural del agua después de la influencia del hombre. El crecimiento continuo de la población alrededor del planeta hace del tratamiento de aguas residuales un requerimiento y un desafío a la vez. Las estaciones depuradoras de aguas residuales (EDAR; acrónimo en inglés*, WWTP*) tienen que tratar caudales más elevados con las mismas instalaciones construidas años atrás. La necesidad de construcción de nuevas EDAR en áreas más reducidas es otro de los puntos que requiere una solución. Con este fin, los reactores discontinuos secuenciales (RDS; acrónimo en inglés*, SBR*) han sido el punto de mira de diferentes estudios y aplicaciones gracias a su reducido impacto en lo que a utilización de espacio se refiere. Además, estos reactores son extremadamente flexibles y adaptables a cambios de operación para la regulación de efluentes, como seria la eliminación de nutrientes. Sin embargo, la problemática de las limitaciones en cuanto a espacio y el tratamiento de altas cargas dirigen la investigación a sistemas más compactos.

La eliminación biológica de nutrientes (EBN; acrónimo en inglés*, BNR*) ha sido investigada durante décadas. Por otro lado, la continua demanda de agua conduce a las EDAR a trabajar a su máxima capacidad. El incremento de carga conlleva eventualmente a una desestabilización de la población microbiana y, en consecuencia, a la pérdida del proceso de eliminación de nutrientes. Cuando la perturbación de las reacciones biológicas se produce en un mismo reactor, la recuperación de la estabilidad del sistema requiere diferentes condiciones de operación para la eliminación de nutrientes como el nitrógeno y el fósforo. Operando con los dos procesos de manera simultánea requiere una solución de compromiso en cuanto a las condiciones de trabajo. Por esta razón, diferentes cambios consecutivos de operación son necesarios para cada nutriente cuando aparece una desestabilización del sistema antes de acoplar todas las reacciones biológicas. Con este fin, la presente tesis describe un protocolo de trabajo para la recuperación y estabilización de la eliminación de nitrógeno y fósforo después de un incremento de carga. El estudio enfoca diferentes condiciones de operación como la estrategia de alimentación o la distribución en la duración de las fases. La nitrificación debería ser el primer proceso a ser

recuperado, ya que se ve fuertemente afectado por una edad del lodo baja, posible consecuencia del aumento de carga a tratar. Alargar la fase aerobia a cuenta de una parte de la fase anaerobia, pero aún más aplicar largos periodos aerobios evitando el tiempo de respuesta entre fases, mejoraría el proceso de nitrificación. Cuando la oxidación del amonio aumenta, la concentración de nitratos crece en el reactor. La eficiencia de desnitrificación, pues, será el próximo objetivo a tener en cuenta, ya que los nitratos son el interferente principal en la eliminación del fósforo debido a la competencia de los dos procesos para la materia orgánica. Cuando se trabaja con una estrategia de alimentación por etapas, una distribución de volumen mayor en la primera alimentación en el inicio del ciclo reducirá la cantidad de nitratos a la salida del reactor. Del mismo modo, aumentará la cantidad de materia orgánica disponible para el proceso de eliminación biológica de fósforo. Finalmente, como último paso de mejora, las fases anaerobias deberán ser suficientemente largas para estabilizar la captación de materia orgánica y aumentar la bioacumulación de fósforo.

En referencia a la compactación y reducción de la producción de biomasa activa se refiere, el lodo granular aerobio ha sido propuesto durante los últimos años como alternativa para el tratamiento de aguas residuales. Comparado con los sistemas convencionales de lodos activos, estos microorganismos encapsulados permiten una acumulación de grandes cantidades de biomasa activa en un solo reactor, presentando una estructura microbiológica más densa y una mejor sedimentabilidad. Todas estas características permiten que el lodo granular aerobio sea propuesto como una alternativa viable para la reducción del espacio de las depuradoras y para el tratamiento de cargas más elevadas. En contraposición, el proceso de granulación tiene todavía muchos parámetros desconocidos que son necesarios investigar. Además, para la formación de sistemas granulares en aguas residuales de baja carga, como por ejemplo las aguas urbanas que presentan cargas de materia orgánica menores a 1 Kg $DQO \cdot m^{-3} d^{-1}$, el proceso de granulación se ralentiza y son necesarios periodos más largos para llegar al estado estacionario.

En la presente tesis se han llevado a cabo estudios comparativos de granulación en aguas residuales sintéticas y reales con concentraciones similares a las

urbanas. Primeramente, una estrategia de reducción del tiempo de sedimentación fue aplicada para seleccionar los agregados de biomasa con sedimentación más rápida. Esta metodología fue llevada a cabo en reactores idénticos, tratando diferentes volúmenes de agua residual. Se observó que relaciones volumétricas de cambio de 40-50% mejoraban la formación del gránulo, en contraposición a relaciones de cambio mayores, aunque éstas incrementaran la carga aplicada sobre el sistema. Este hecho fue debido principalmente a un mayor lavado de la biomasa, afectando al proceso de granulación y al rendimiento de eliminación de nutrientes. Uno de los principales problemas del tratamiento de agua residual es el bajo contenido de material orgánico soluble. Por lo que a los reactores granulares se resfiere, el uso de agua residual sin pretratar en vez de agua residual sedimentada o decantada puede favorecer la agregación de biomasa flocular en partículas, ya que aporta cargas orgánicas más elevadas.

En lo que la EBN en lodo granular se refiere, los microorganismos se sitúan el mismo agregado de biomasa en vez de en diferentes reactores. Por esa razón, las bioreacciones del nitrógeno y el fósforo tienen unas interacciones más fuertes entre ellas. El proceso simultáneo de nitrificación y desnitrificación, a veces junto a la eliminación biológica de fósforo, se ha observado en el tratamiento de aguas residuales de baja carga debido a la difusión del oxígeno en los gránulos. La influencia del nitrito y el nitrato como productos de la nitrificación se evaluó en procesos simultáneos de lodo granular. El nitrito a la vez que el nitrato era viable para la eliminación simultánea de nitrógeno y fósforo, aunque a igual concentración de los dos, el nitrato permitía una mayor eliminación de fósforo. Contrariamente, la nitrificación-desnitrificación usando nitrito como producto intermedio permitía una reducción de los requerimientos de materia orgánica. Sin embargo, debe tenerse en cuenta la concentración de nitrito ya que la eliminación de fósforo puede ser inhibida en presencia de elevadas concentraciones de ácido nitroso. Así pues, el nitrito puede mejorar la eliminación simultánea en gránulos cuando los agregados presenten un tamaño suficientemente grande para permitir la difusión de nutrientes en el centro y, así, prevenir los procesos de inhibición.

INTRODUCTION

Chapter 1. Introduction

This chapter gives an overview of the thesis carried out and summarizes the information most relevant to this research from the literature regarding biological processes for wastewater treatment, SBR technology and granular technology.

1.1 Research motivation

Nutrient removal is one of the key issues in mantaining the natural water cycle unaffected after human influence. Nitrogen and phosphorus removal is one of the objectives of wastewater treatment under the European Water Framework Directive (2000/60/EC). Biological nutrient removal (BNR) has been investigated for several decades. However, most of the Spanish wastewater treatment plants (WWTPs) still don't apply combined biological nitrogen and phosphorus removal processes (National Plan of Water Quality: Treatment and Sanitation 2007-2015). Instead, they use chemical precipitation (i.e., $FeCl_3$ addition) for phosphorus removal, which increments the treatment cost because of the chemical products used and the handling of the excess of contaminated sludge that is produced.

Continued population growth in all around the world makes domestic wastewater treatment a requirement and, at the same time, a challenge. Higher flows have to be handled in WWTPs using facilities built years ago or new designs with reduced surface areas. Sequencing batch reactors (SBRs) have been studied and used for years because of their relatively small footprint. In addition, they are extremely easy to adapt to regulatory changes for effluent parameters such as nutrient removal (EPA, 1999). However, even more compact systems than SBRs are required to overcome space limitations and deal with high loading treatments.

Granular sludge has been proposed in recent years as an alternative for wastewater treatment (Adav *et al.*, 2008a). Compared to conventional activated sludge, these self-immobilized microorganisms allow the accumulation of large amounts of active biomass in the reactor with a dense, strong microbial structure and good settleability. Furthermore, they are able to withstand high organic loading rates (Morgenroth *et al.*, 1997; Beun *et al.* 2002a; Liu and Tay, 2004; Adav *et al.*, 2010b). All these characteristics make aerobic granules a reliable alternative to reduce space and increase loading rates in treatment plants. Despite all its advantages, however, granular sludge has usually been developed at high organic loading rates for organic matter removal. Some studies have been done on granular treating low-strength

wastewater, but not many have been applied to nitrogen and phosphorus removal (de Kreuk and van Loosdrecht, 2006; Li *et al*., 2007; Ni *et al*., 2009). There is still a lack of knowledge in the granular sludge field about treating domestic wastewater for nutrient removal purposes.

This thesis follows one of the research lines of the LEQUIA-UdG group (Laboratory of Chemical and Environmental Engineering, University of Girona; Institute of the Environment), which focuses all its efforts on environmental engineering solutions and applications. In terms of domestic wastewater treatment, three PhD theses during the last decade have focused on the control and optimization of nitrogen removal and, most recently, biological phosphorus removal (Vives, 2004; Corominas, 2006; Puig, 2008). Using the knowledge base of the group, the present thesis focuses on the enhancement of BNR processes to obtain cost efficient systems in terms of treatment and construction. Firstly, solutions for nitrogen and phosphorus removal recovery in the same reactor were investigated. Later, the formation and operation of a granular sludge system treating domestic wastewater was studied to improve both the performance and the compactness of a BNR system.

1.2 Literature review

1.2.1 Activated sludge systems

Biochemical processes, in which chemical transformations are carried out by living microorganisms, have many diverse uses and one of them is the treatment of wastewater. The most common operation, activated sludge, was given its name even before its biochemical nature was recognized in the early 1900s (Grady and Lim, 1980).

Methods for purification in secondary treatment units, like activated sludge systems, are similar to the *self-purification processes* that occur naturally in rivers and streams, and involve many of the same organisms (Gray, 2004).

By definition, the basic activated-sludge treatment process, as illustrated in Figure 1.1, consists of the following three basic components: (i) a reactor in which the microorganisms responsible for treatment are kept in suspension; (ii) a liquid-solid separation component, usually in a sedimentation tank; and (iii) a recycling system for returning solids (biomass) to the reactor (Tchobanoglous *et al.*, 2003). Two outputs are obtained from the activated-sludge system: treated wastewater and waste sludge.

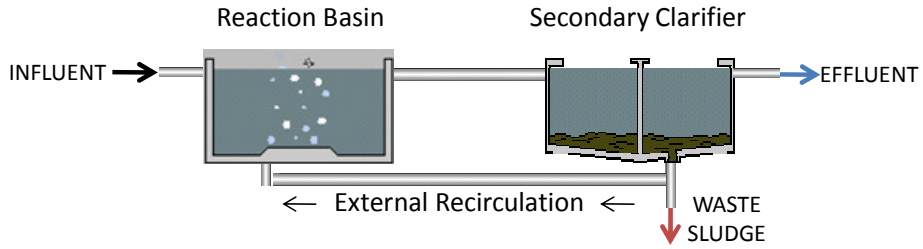

Figure 1.1. Typical activated sludge process scheme.

Since the process came into common use, activated sludge systems have been designed for organic matter removal. However, WWTPs have been modified over the years to comply with standard regulations and improve biological nutrient (nitrogen and phosphorus) removal under different conditions in the reactor.

1.2.2 Organic matter removal

The removal of organic matter is carried out by heterotrophic microorganisms, predominantly bacteria but also occasionally fungi. The microorganisms break down the organic matter by two distinct processes: biological oxidation (Equation 1.1) and biosynthesis (Equation 1.2), both of which result in the removal of organic matter (Gray, 2004).

Particulate and colloidal organic carbon must be hydrolyzed first before the bacteria can use it for their metabolism. Particulate biologically non-degradable carbon is incorporated in the activated sludge flocs and removed from the system with the excess sludge (Figure 1.1).

$$\underset{(organic\ matter)}{COHNS} + O_2 + bacteria \rightarrow CO_2 + NH_3 + other\ end\ products + energy$$

Eq. 1.1

$$\underset{(organic\ matter)}{COHNS} + O_2 + bacteria \rightarrow C_5H_7NO_2\ (new\ cells)$$

Eq. 1.2

Even though classical removal takes place under aerobic conditions, organic matter can be oxidized through other mechanisms related to nutrient removal. Heterotrophic organisms are able to oxidize organic carbon using nitrite or nitrate as an electron acceptor (denitrification) under anoxic conditions. Furthermore, when biomass is exposed to high concentrations of organic substrates followed by a period without any external carbon source, some heterotrophic bacteria are also able to accumulate substrate such as internal storage products as glycogen or polyhydroxyalkanoates (PHAs). Organisms can use the storage substrates as energy pools during famine periods (van Loosdrecht *et al*., 1997a; Reis *et al*., 2003). A special way of storing organic carbon before it is used in the metabolism is performed by phosphorus-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) (de Kreuk, 2006).

1.2.3 Nitrogen removal

Nitrogen (N) can be removed from streams using different methods such as physical-chemical treatments, fully autotrophic systems (partial nitritation - anammox), and conventional nitrification-denitrification processes (López, 2008; Ganigué, 2010).

Low-strength wastewater is considered a non-complex influent and the conventional nitrification/denitrification process is the most widely applied method in these currents. In the first stage, **nitrification**, ammonium is oxidized to nitrite (nitritation; Equation 1.3) and then further to nitrate (nitratation; Equation 1.4) in a two-step biochemical reaction carried out by autotrophic bacteria, namely ammonium- and nitrite-oxidizing bacteria (AOB and NOB,

respectively). This process has to be accomplished under aerobic conditions (Tchobanoglous *et al.*, 2003).

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O \qquad \text{Eq. 1.3}$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \qquad \text{Eq. 1.4}$$

Nitrification has traditionally been considered as a single process in conventional WWTPs. In fact, ammonium oxidation is the rate-limiting step while nitrite is usually consumed at the same time it is produced (Ganigué, 2010). However, different parameters such as pH, temperature and oxygen concentration, and others less important in domestic wastewater because of low concentrations (free nitrous acid (FNA, HNO_2), free ammonia (FA, NH_3), toxics and salinity) can cause inhibition problems or a decoupling of AOB and NOB and, as a consequence, an accumulation of nitrite in the system.

The second stage of nitrogen removal consists of the **denitrification** process, which is based on the biological oxidation of many organic substrates in wastewater treatment, using nitrate or nitrite as the electron acceptor instead of oxygen. The nitrate reduction reactions involve the following reduction steps from nitrate to nitrite, to nitric oxide, to nitrous oxide, and finally to nitrogen gas, as shown in Equation 1.5:

$$NO_3^- \xrightarrow{OM} NO_2^- \xrightarrow{OM} NO \xrightarrow{OM} N_2O \xrightarrow{OM} N_2 \qquad \text{Eq. 1.5}$$

The denitrification process releases alkalinity in the media, which increases the pH. However, the whole nitrification-denitrification system reduces the need for chemicals to control pH, as the nitrification process causes a pH decrease while denitrification increases it (Tchobanoglous *et al.*, 2003).

In order to accomplish nitrogen removal, WWTPs were modified by adding an anoxic basin at the beginning of the configuration. A modified Ludzack-Ettinger configuration used for organic matter and nitrogen removal is depicted in Figure 1.2. Internal recirculation supplies the nitrate generated during

nitrification (aerobic basin) and denitrification occurs thanks to the organic matter provided from the wastewater.

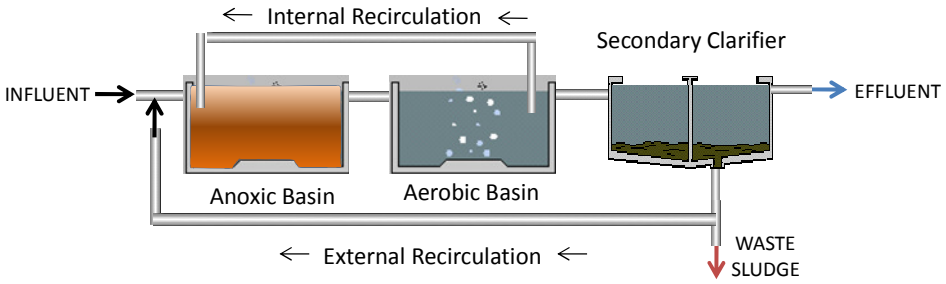

Figure 1.2. Modified Ludzack-Ettinger configuration for organic matter and nitrogen removal.

1.2.4 Phosphorus removal

Phosphorus (P) is a key nutrient that stimulates the growth of algae and other photosynthetic microorganism such as toxic cyanobacteria, and must be removed from wastewater to avoid eutrophication in aquatic water systems (Oehmen *et al*., 2007). The traditional method for removing phosphate from wastewater is by the addition of precipitating chemicals (iron, aluminum or lime) to the media. In general, efficient phosphorus removal is obtained, but large amounts of sludge are generated. Furthermore, the concentration of chemicals in the effluent has a negative effect due to possible toxicity and the treatment cost is increased due to precipitants and sludge volume management (Puig, 2008).

The removal of phosphorus by biological means is known as enhanced biological phosphorus removal (EBPR). The process is based on a bioaccumulation of phosphorus, which immobilizes the nutrient inside the cells. The group of organisms that are largely responsible for EBPR are PAOs. These organisms are able to store phosphate as intracellular polyphosphate, leading to phosphorus removal from the bulk liquid phase via PAO cell removal in the waste activated sludge (Oehmen *et al*., 2007).

EBPR is achieved by combining anaerobic and aerobic conditions (Figure 1.3). During the initial anaerobic conditions (in the absence of oxygen, nitrite or nitrate), PAOs are able to assimilate readily biodegradable organic matter (mostly volatile fatty acids – VFAs - which are fermentation products) and store them as PHAs. The reducing power for these processes is obtained by the glycolysis of internally stored glycogen in accordance with the Mino model (Mino *et al.*, 1987). The energy is obtained through the hydrolysis of their intracellular stored polyphosphate (PolyP) with a consequent phosphate release into the bulk liquid (Figure 1.3, left). In the second aerobic phase, PAOs use their previously stored PHA as the energy source for biomass growth and glycogen and PolyP replenishment. As a result, phosphate is removed from the media and bioaccumulated within the cells as storage products. P removal is accomplished due to a higher aerobic phosphate uptake than anaerobic phosphate release (Figure 1.3, right).

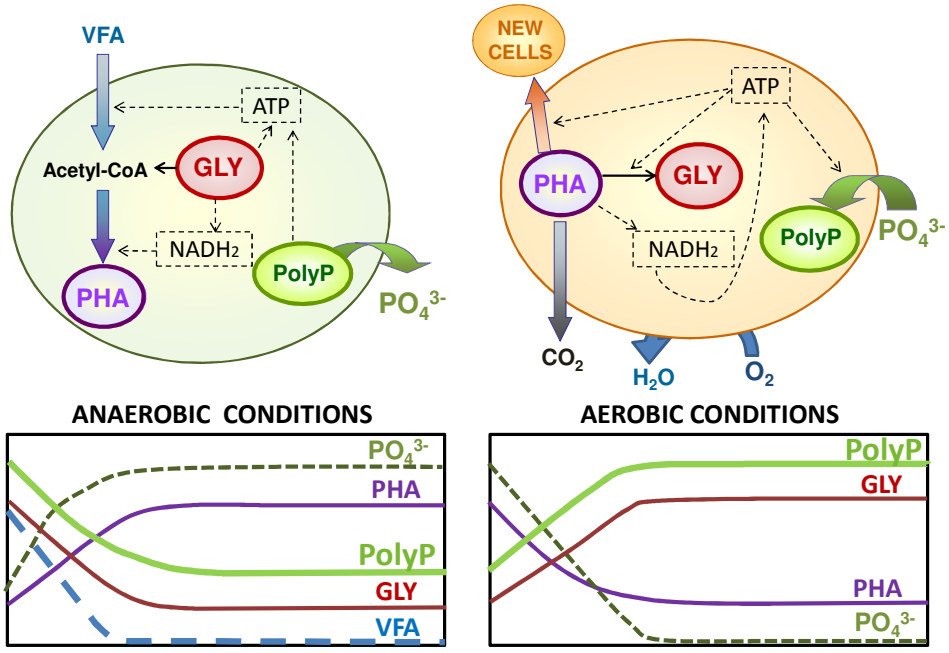

Figure 1.3. Schematic representation of anaerobic (left) and aerobic (right) PAO metabolism. Graphs depict the profile concentrations of storage compounds (solid lines) and bulk liquid compounds (dashed lines).

Anaerobic conditions have been applied in EBPR systems to obtain PAO enriched cultures. However, it has been seen that phosphate removal can significantly deteriorate in some cases with the application of anaerobic-aerobic conditions. This breakdown of the process is mainly due to the growth of a group of microorganisms capable of producing PHA through VFA assimilation via glycolysis as the sole energy source. This group of bacteria was named GAO to differentiate them from those that promote EBPR (Mino *et al.*, 1995; Liu *et al.*, 1996). GAOs are able to compete with PAOs for carbon sources but without taking up phosphorus from the media (Figure 1.4).

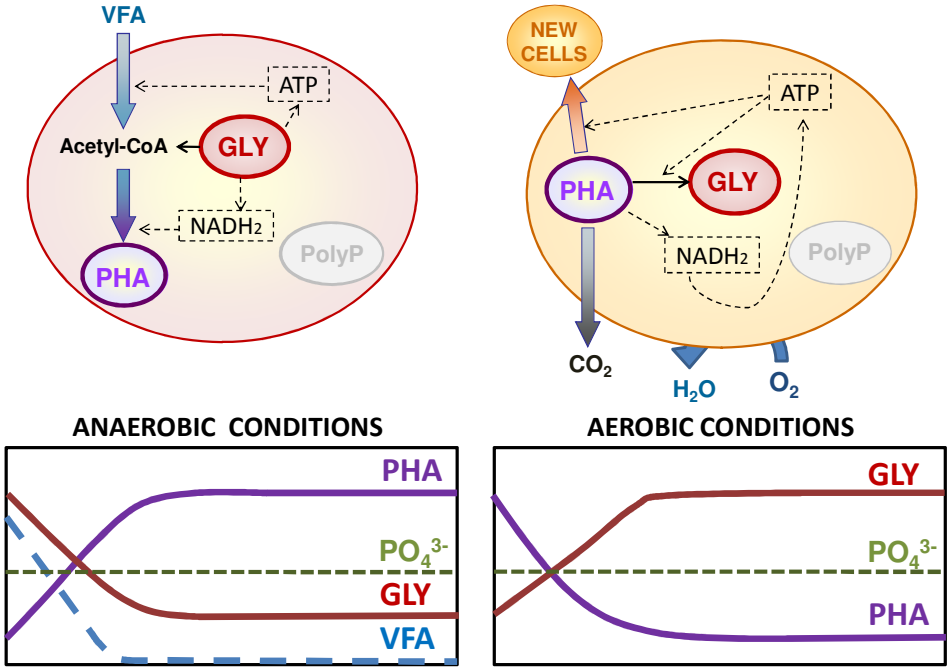

Figure 1.4. Schematic representation of anaerobic (left) and aerobic (right) GAO metabolism. Graphs depict the profile concentrations of storage compounds (solid lines) and bulk liquid compounds (dashed lines).

The term 'PAO' or 'GAO' can include multiple specific microbial groups. The most well known PAO group is *Candidatus Accumulibacter Phosphatis*, which consists of two types with metabolically distinct features such as denitrification capacity (see Section 1.2.5: Simultaneous processes) and anaerobic utilization of the tricarboxylic acid (TCA) cycle (Zhou *et al.*, 2009). Another group of PAOs

within the *Actinobacteria* group have been found to be abundant in domestic wastewater (Kong *et al*., 2005). Even though they do not store PHA and prefer to take up amino acids rather than VFAs, they are an important contributor to P removal. Regarding GAOs, Phylogenetic two groups have also been observed, *Candidatus Competibacter* and *Defluviicoccus Vanus*, both of which have several sub-groups with varying denitrification capabilities but similar anaerobic stoichiometry (Oehmen *et al*. 2010).

Successful operation of the EBPR process depends on numerous operational factors like pH, aeration, nitrite and nitrate concentrations, temperature and the carbon source supplied during anaerobic conditions. Some of these factors directly affect microbial PAO-GAO competition. To favor PAO over GAO activity, optimal operational conditions for EBPR systems are: pH higher than 7.5 (Smolders *et al*., 1994; Filipe *et al.,* 2001; Jeon *et al*., 2001); avoidance of overly aerated systems (Brdjanovic *et al*., 1998) and high dissolved oxygen set-points (Lemaire *et al.* 2006); operation at a temperature of 20ºC or below (Whang and Park, 2002; López-Vázquez *et al*., 2007); no nitrate in the anaerobic zone to avoid organic matter competition (Puig *et al*., 2007a); low nitrite concentration during phosphorus uptake to prevent nitrite or FNA inhibition (Meinhold *et al.,* 1999; Saito *et al.,* 2004; Zhou *et al*., 2007).

With regard to the carbon source, in early studies authors constantly referred to good EBPR activity when using carbon sources belonging to the VFA group. The most prevalent VFA in EBPR plants is acetate, though in plants where prefermentation is employed, propionate is often present in significant quantities. The use of acetate has often been documented as yielding robust and stable P removal performance, but there are also many reported occasions where the P removal deteriorated due to microbial competition between GAOs and PAOs (Oehmen *et al*., 2007). In this connection, the use of propionate has been proposed as a suitable carbon source for long-term EBPR systems, as it results in better net P removal and enriched biomass by PAO over GAO (Chen *et al*., 2004; Oehmen *et al.*, 2006; Pijuan *et al*., 2009).

It must be remembered that wastewater is a heterogeneous and complex influent which often reaches the treatment plant without complete acid

fermentation (Pijuan, 2004) and containing large amounts of non-VFA compounds. To account for complex substrates for P removal, different studies have been carried out into treatment with glucose (Carucci *et al.*, 1996; Jeon and Park, 2000), malate or lactate (Satoh *et al.*, 1996), starch (Randall *et al.*, 1994), mixtures of acetate and peptone (Liu *et al.*, 1997) and ethanol (Puig *et al.*, 2008) among others. The experimental evidence is such that PAOs appear to have mechanisms that allow the use of non-VFA compounds without prior fermentation by heterotrophic bacteria (Pijuan, 2004).

Finally, with regard to biological phosphorus removal in WWTPs, different configurations have been designed and applied based on anaerobic-aerobic zones (i.e., the Phoredox process). When nutrient treatment is employed, a combination of anaerobic, anoxic and aerobic conditions is required for both nitrogen and phosphorus removal. Configurations such as the A^2/O process (Figure 1.5), the modified Bardenpho^{TM}, the Virginia initiative plant (VIP) process, the PhoStrip process and the university of Cape Town (UCT) process have all been applied for biological nutrient removal (Puig, 2008).

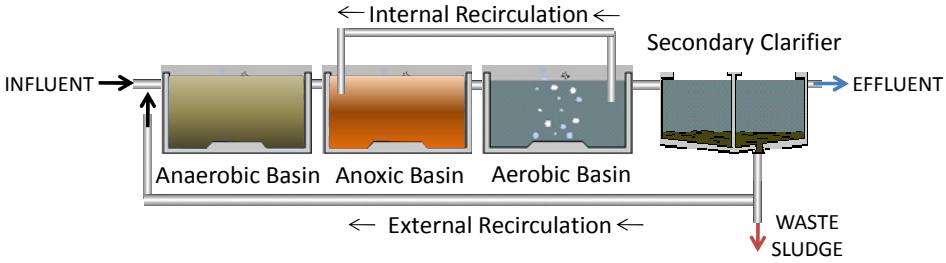

Figure 1.5. A^2/O configuration for organic matter, nitrogen and phosphorus removal

1.2.5 Simultaneous processes

When different microorganisms coexist in the same tank, simultaneous nitrification-denitrification (SND) can take place as a result of an existing oxygen gradient, with denitrification occurring in the areas of low dissolved oxygen (DO) concentrations (Keller *et al.*, 1997; Seviour and Blackall, 1999). Nitrate is usually the end product of nitrification, but of particular interest in the SND process is the fate of nitrite, which is an intermediate of both

nitrification and denitrification (Equation 1.3 and 1.5). For the most effective nitrogen removal, any nitrite that is produced by nitrification should be reduced to N_2 rather than oxidized to nitrate, which represents an unnecessary use of oxygen (25% more of O_2 savings; Gibbs *et al*., 2004) and a reduction of nearly 40% of the organic matter demand for denitrification (Tchobanoglous *et al*., 2003).

While the majority of P removal from the EBPR process is often achieved through anaerobic-aerobic cycling, other simultaneous processes are achieved by denitrifying PAOs (DPAOs) in anaerobic-anoxic EBPR systems, where the removal of nitrite and nitrate (NO_x^-) and P happens at the same time (Lemaire *et al*., 2008a). DPAOs are considered a fraction of PAOs which can use nitrate and/or nitrite as electron acceptor instead of oxygen (Figure 1.6) (Kerrn-Jespersen *et al*., 1994; Kuba *et al*., 1996).

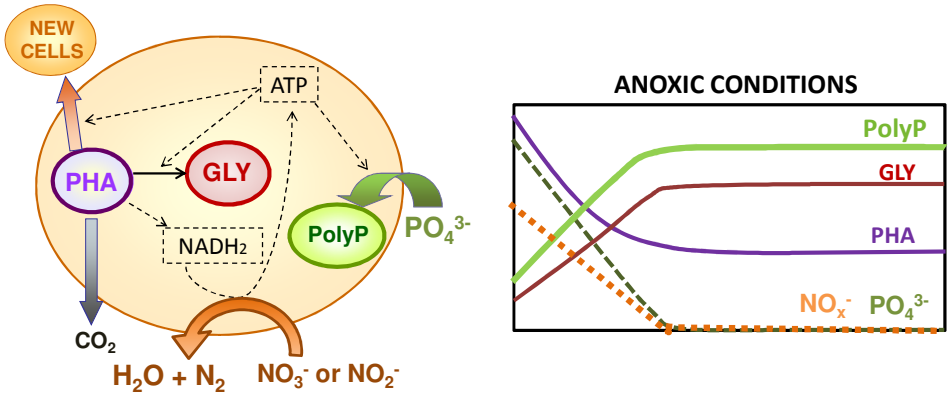

Figure 1.6. Schematic representation of anoxic DPAO metabolism. Graphs depict the profile concentrations of storage compounds (solid lines) and bulk liquid compounds (dashed lines).

Batch experiments carried out by Carvalho *et al*. (2007) suggest the existence of different types of PAOs: one group able to use nitrate directly as electron acceptor and another group that might be able to use nitrite, but not nitrate. However, some of the PAO population are not able to use either nitrate or nitrite (Meinhold *et al*., 1999). In some studies, it was reported that the microorganisms able to uptake phosphorus under aerobic conditions are the same to those that can do it under anoxic conditions. Zeng *et al.* (2003a)

showed that Accumulibacter were the dominant population in both anaerobic-aerobic SBR and anaerobic-anoxic SBR. The aerobic population needed few hours for nitrate acclimation and synthesizing the corresponding enzymes, indicating that these PAO and DPAO were the same microorganisms. The alternation of denitrifying and aerobic periods before the activated sludge is exposed to anaerobic conditions could be the reason for *Accumulibacter* to adapt to quick changes in the electron acceptor conditions and thus express the relevant enzymes (Vargas, 2010). As a result of this adapting period to denitrifying conditions, the rate of P uptake by PAOs under anoxic conditions is generally lower than under aerobic conditions. However, substantially less chemical oxygen demand (COD) is required for this process as compared to separate P and N removal, which together with the savings in aeration resulting from the use of NO_x^- instead of oxygen, makes denitrifying P removal economically very attractive (Oehmen *et al*., 2007). As in the PAO case, DPAO also has a competitor organism (denitrifying GAO or DGAO) which is able to use nitrite or nitrate as electron acceptor without any P removal activity (Zeng *et al*., 2003b).

In order to obtain an even more optimized system, EBPR and SND processes can be amalgamated into one process called simultaneous nitrification, denitrification and phosphorus removal (SNDPR) (Zeng *et al*., 2003c; Yilmaz *et al*., 2008). This process is based on the application of anaerobic-aerobic conditions working at low oxygen concentrations. During anaerobic conditions, COD is taken up and P is released. Afterwards, during aerobic conditions, ammonium is oxidized to nitrite or nitrate and these compounds are reduced simultaneously with P by DPAO activity (Lemaire *et al.,* 2008).

1.2.6 SBR technology

Nutrient removal in WWTPs is usually accomplished in continuous systems in which the biological treatment happens in a reaction basin and the sludge is separated from the treated water in a settler (Figure 1.1). With the improvement of BNR, WWTP configurations have changed over the years (see Figure 1.2 and Figure 1.5) with a consequent increase in the land requirements of treatment facilities.

Sequencing batch reactor (SBR) technology is a fill-and-draw activated sludge system where all reactions and sludge separation can take place in the same basin (Figure 1.7). An SBR converts the conventional wastewater treatment process from space-course to time-course, which substantially reduces space occupation (Tchobanoglous *et al.*, 2003). SBRs allow greater flexibility for adapting phases required for nutrient removal according to the influent variations (EPA, 1999). However, more sophisticated equipment is necessary for their operation and automation (Corominas, 2006).

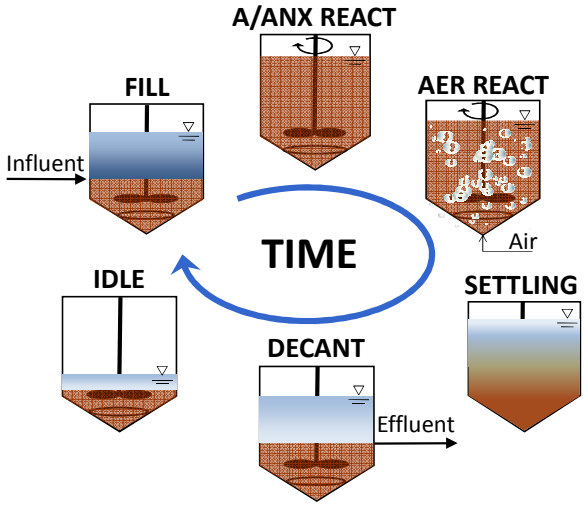

Figure 1.7. Sequence of phases in the SBR operation. A/ANX REACT and AER REACT stand for anaerobic/anoxic and aerobic reaction.

SBRs operate applying cycles that are repeated over time. A cycle is built with a sequence of phases according to the main objective desired. The **fill phase** consists of the introduction of the influent wastewater into the reactor. This can be accomplished under different conditions: static or mixed; anaerobic, anoxic or aerobic phases; fed batch strategy (continuous filling throughout the reaction) or step-feed strategy (filling by pulses during the reaction sequence) (Irvine and Ketchum, 1988). The **react phase** is usually carried out under mixing conditions and can be anaerobic, anoxic or aerobic according to the purpose of the cycle. During the **settling phase**, all equipment is maintained in the off-mode to let the sludge separate from the wastewater. Sludge wasting in order to control the age of the biomass can be carried out in the reaction or settling

phases. After settling, treated water is discharged in the **decant phase**. The **idle phase** is the time between cycles. It is used to adjust times between SBRs in multitank systems, but can be omitted.

1.2.7 Granular sludge

Granular sludge was first developed in an anaerobic process and resulted in much more compact systems. However, factors affecting anaerobic granulation are not well understood (Schmidt and Ahring, 1996; Lettinga *et al*., 1997). Since granulation was studied almost entirely in the context of methanogenic (anaerobic) systems, it was regularly hypothesized that specific bacterial interactions in this process were the main cause of the granule formation (Beun *et al.*, 1999). It was not until at the end of the 1990s that granules became a revolutionary technology applied in aerobic systems (Morgenroth *et al*., 1997; Beun *et al*., 1999; Dangcong *et al*., 1999). As Figure 1.8 shows, research into anaerobic granular sludge (AnGS) has been going on for a long time, while interest in aerobic granular sludge (AGS) has increased worldwide during the last decade.

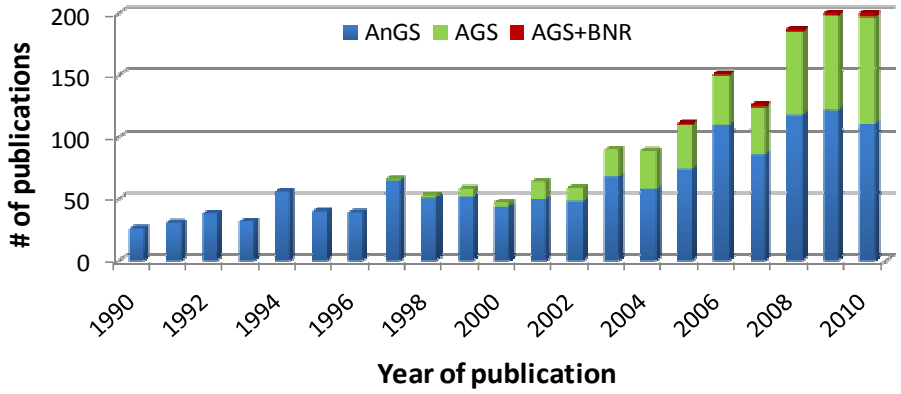

Figure 1.8. Number of publications per year of anaerobic granular sludge (AnGS), aerobic granular sludge (AGS) and AGS applied to biological nutrient removal (AGS+BNR). (SciVerse Scopus, Elsevier®, December 2010).

It must be pointed out that although the application of this technology to BNR (AGS + BNR) has not been completely successful in the last few years, it is still a hot topic in recent research (Gao *et al*., 2011; Zhang *et al*., 2011). Studies into

granular sludge applied to a wide variety of wastewater have concluded that the more diluted the influent (i.e., domestic wastewater), the worse the granulation performance. Tested systems that alternated anaerobic and aerobic periods for granulation with low-strength wastewater required a longer start-up time (de Kreuk *et al.,* 2007). For this reason, there has been very limited study of granulation treating municipal wastewater and the few investigations that have been done do not focus on nitrogen and phosphorus removal.

According to the definition agreed in the first and second Aerobic Granular Sludge Workshop (Munich, Germany, 2004; Delft, The Netherlands, 2006):

> "*Granules making up aerobic granular activated sludge are to be understood as aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs*".

The implication of this is that granules contain active microorganisms without carrier material and not only components of microbial origin (such as proteins, EPS, etc.), they do not coagulate as floccular sludge during settling, they present a SVI_{30}/SVI_{10} ratio (sludge volumetric index at 10 and 30 minutes) close to one and they have a minimum size of 0.2 mm (de Kreuk *et al*., 2007).

Granular formation is one of the key points of the process. The causes and mechanism of granulation are not yet fully understood, but several important factors have been described in the literature (Figure 1.9), including substrate composition, organic loading rate, feast-famine conditions, reactor design, settling time, volumetric exchange ratio (VER), aeration intensity (hydrodynamic shear force), intermittent feeding and SBR operation among others (Liu and Tay, 2004; Adav *et al*., 2008a).

Granules are well known to be fast settling particles. This advantage reduces the volume of the settlers or even avoids the use of them because sludge separation is integrated in the reactor itself. An extra advantage of the application of granules in SBRs because of its discontinuous characteristics

(Adav *et al.*, 2008a) is that, thanks to their high settling velocity (Beun *et al.*, 1999), the settling phase requires less time.

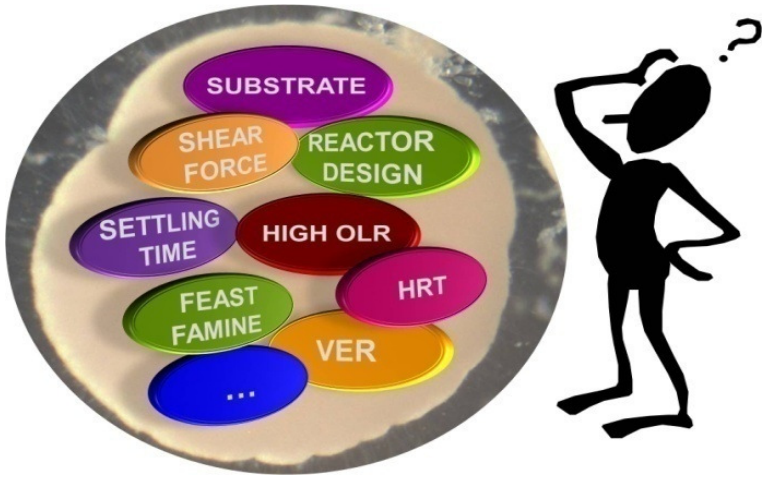

Figure 1.9. Different parameters affecting granulation still under research.

Granular sludge reactors are also desirable in biological treatment processes because a very high biomass concentration can be achieved in the treatment systems. This high concentration means that contaminant transformation is rapid, and high organic loading rates or large volumes of waste can be treated in reactors which take up only a small amount of space (Liu and Tay, 2002; Adav *et al.*, 2010a). Furthermore, their compact structure and their higher density compared to conventional activated sludge gives granules a stronger microbial structure, makes them more tolerant to toxicity and allows simultaneous reactions (i.e., SNDPR) thanks to diffusion within the aggregate (Zeng *et al.*, 2003c; Adav *et al.*, 2008a; Lemaire *et al.*, 2008).

1.2.7.1 Reactor operational conditions

Aerobic granulation has been mainly reported in SBRs (Liu and Tay, 2004; Adav *et al.*, 2008a). The major selection factors responsible for aerobic granulation have been identified as the settling time and exchange ratio (Liu *et al.*, 2005). For this reason, the application of short settling times is generally used to granulate the most common kind of sludge under aerobic conditions, with the

purpose of selecting bioparticles according to their settling velocity. McSwain *et al*. (2004a) found that in short-term experiments, granules can form after the application of both 2 and 10 minutes of settling time. Even though shorter settling times resulted in a greater washout of the biomass (down to 0.7 g MLSS·L^{-1}), a fully granulated sludge with better settling properties, that reached a pseudo-steady-state after long-term operation, was obtained.

Besides short settling times, organic loading rates (OLRs) and feed pattern conditions are some of the key operational parameters in granular sludge formation. High OLRs of between 2 and 20 Kg COD·$m^{-3}d^{-1}$, depending on the substrate, are commonly applied for different removal purposes due to ease of aerobic granulation and later treatment (Moy *et al*., 2002; McSwain *et al*., 2004b; Schwarzenbeck *et al*., 2004; Zheng *et al*., 2006). However, Adav *et al*. (2010a) found that granules started to disintegrate when OLRs over 20 Kg COD·$m^{-3}d^{-1}$ were applied, due to the reduced protein quantity secreted by isolates. In contrast, Tay *et al.* (2004) reported that it was difficult to form granules with an OLR lower than 2 Kg COD·$m^{-3}d^{-1}$. The study showed that the lower OLR resulted in slower formation of granules and a longer time being taken to reach a steady state. Therefore, a considerably longer start-up time was needed when treating domestic wastewater (de Kreuk, 2006).

With regards to feed pattern conditions, spontaneous aerobic granulation of suspended growth can be obtained in an SBR by applying short fill periods. In order to form spherical compact granules, the conversion of biodegradable substrate into inter-cellular stored substrate must occur rapidly in the reactor. This is often described as a feast-famine regime (Figure 1.10), and can easily be applied in an SBR with short fill periods (McSwain *et al*., 2004b). During the feast period the organic matter is oxidized and stored inside the bacteria cells as glycogen, lipids and PHA (van Loosdrecht *et al*., 1997a), while during the famine period the bacteria grow on the stored compounds (Beun *et al*., 2002b). The application of this feeding strategy is supposed to prevent competition between flocs and filamentous bacteria, which usually outgrow flocs when substrate concentrations in the bulk are low. A feast-famine regime lowers the maximum growth rate during the famine phase, with the organisms using slowly biodegradable internally stored substrate (PHA) to grow in this period.

This feeding regime allows the formation of stable granular sludge (de Kreuk and van Loosdrecht, 2004). Furthermore, other studies have observed that bacteria become more hydrophobic under periodic feast-famine conditions, which facilitates microbial aggregation (Campos *et al*., 2009).

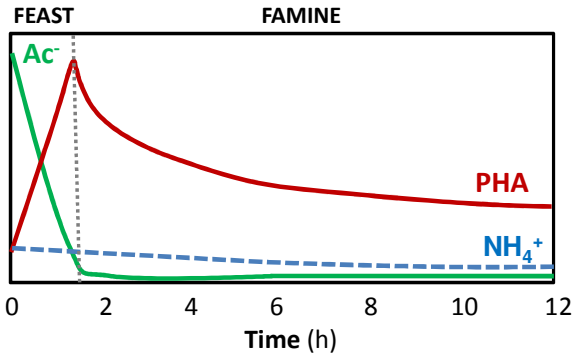

Figure 1.10. Changes in concentrations and rates during a feast-famine regime. Organic matter is consumed for growth and PHA storage during feast phase. PHA is used as energy source for growth during famine conditions (based on Beun *et al*., 2002b).

1.2.7.2 Environmental conditions

Further research has been carried out to find operational conditions for granular sludge. However, literature reviews have suggested that some environmental parameters also affect granule formation (Liu and Tay, 2004; de Kreuk *et al*., 2007; Adav *et al*., 2008a; Campos *et al*., 2009).

Aerobic granules have been cultivated using a wide range of synthetic wastewater (Liu and Tay, 2004; Adav *et al*., 2008a). It has been postulated that granule microstructure and species diversity is related to the type of carbon source (Campos *et al*., 2009). Information on granule cultivation with real wastewater has also been reported in treating organic matter and nitrogen and even particulate organic matter (Arrojo *et al*., 2004; de Bruin *et al*, 2004; Schwarzenbeck *et al*., 2004; Inizan *et al.,* 2005; Yilmaz *et al*., 2008; Vázquez-Padin *et al*., 2010). Regardless of the origin of the influent, Fang *et al*. (2009) reported that the COD/N ratio had a significant effect on the morphological characteristics of PHB-rich granular sludge. A COD/N ratio of 90 produced an

outgrowth of filamentous bacteria and a consequent disturbance in the settling properties.

The presence of different chemicals in the media has also been investigated over the years. It has been proposed that divalent ions such as calcium (Ca^{2+}) act as a bridge to bind negatively charged groups present on bacterial surfaces and/or extracellular polymeric substances (EPSs) (Campos *et al.,* 2009). A synthetic chelating agent was used by Nancharaiah *et al*. (2008) to enhance granulation in acetate-feed reactors and, in this connection, EPSs as natural chelating agents have been identified and quantified in order to find out their role during granulation and further granule stabilization (Dulekgurgen *et al*., 2003; Wang *et al*., 2005; Zhang *et al*., 2007; Adav and Lee, 2008). McSwain *et al*. (2005) found that granule formation and stability are dependent on a noncellular protein core and that EPS production by slow growing organisms enhances granule formation.

OBJECTIVES

Chapter 2. General Objectives

The problem definition and the general objectives of this thesis are defined in this chapter.

Problem definition

Wastewater treatment plants are under increasing pressure due to the continuous growth in demand for sufficient quantities of good quality water for all purposes. The European Water Framework Directive (EWFD, 2000/60/EC) is a regulatory contribution to the progressive reduction of emissions of hazardous substances in water. With regard to pollution prevention and control, water policy should be based on a combined approach using control of pollution at source through the setting of emission limit values and environmental quality standards. Hence, the Urban Water Directive (91/271/EC) limits the effluent quality in terms of nutrients and, because of that, biological nutrient removal (BNR) has been studied in recent decades with this purpose in mind.

Continuous population growth in all countries makes wastewater treatment a requirement and a challenge at the same time. Higher flows have to be handled within facilities built in smaller surface areas. Sequencing batch reactors (SBRs) have been studied and used for years because of their relatively small footprint. However, even more compact systems are required to overcome space limitations and deal with high loading treatments.

Objectives

The main motivation behind this PhD thesis is to improve BNR in terms of the stability and compactness of the systems. In this light, the principal objective is to evaluate granular sludge formation when treating urban wastewater for BNR. This objective has been divided into the following specific goals:

- Knowledge acquisition of the BNR performance after process disturbance. Study of practical solutions for process recovery.

- Study of different methodologies and operational conditions for granular sludge development in low-strength wastewater.

- Study of the influence of real wastewater on granular sludge formation.

- Long-term nutrient removal performance in granular sludge systems treating domestic wastewater.

- Long-term granular sludge stability.

- Nitrogen and phosphorus interactions in granular sludge systems.

METHODOLOGY

Chapter 3. Materials and methods

This chapter details the experimental set-ups used in this thesis and also summarizes the liquid and solid phase analyses performed. It also deals with biomass characterization and microbiological determination. Finally, it summarizes the calculations applied in some of the other chapters.

3.1 Experimental set-ups

3.1.1 Lab-scale SBRs

The lab-scale sequencing batch reactors (SBRs) used were located in the Laboratory of Chemical and Environmental Engineering (LEQUIA) facilities in the Science and Technology Park of the University of Girona (Girona, Spain). They were mainly composed of three parts: the reactor, the control panel and the refrigerated tank where wastewater was stored. Figure 3.1 shows an image and a diagram of the set-up.

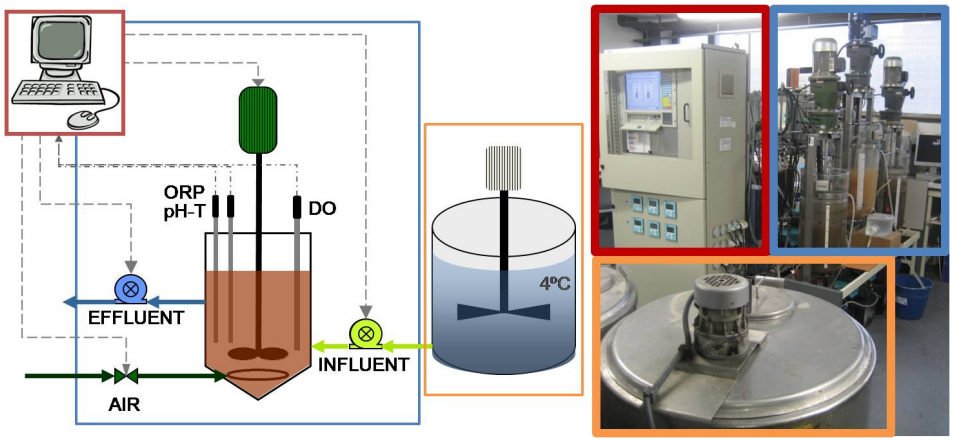

Figure 3.1. Diagram (left) and image (right) of lab-scale SBRs with control (red square), reactor (blue square) and storage tank (orange square) parts.

Each lab-scale SBR (Figure 3.1, blue square) was composed of a cylindrical glass reactor with a maximum volume of 30L. The reactor operated in predefined cycle, repeated over time, at room temperature. Filling and decanting events were achieved using two different peristaltic pumps (Watson-Marlow® 323R/S). Homogeneous conditions during reaction times were attained with a stirring device (a marine helix turning at 190-220 rpm). Aerobic conditions were achieved by injecting compressed air. The dissolved oxygen (DO) set-point was controlled through an ON-OFF control by means of a gas electro-valve. The reactor was equipped with a pH-temperature probe (Endress-Hausser® CPF81-

NN11C2), a oxidation-reduction potential (ORP) probe (Endress-Hausser® CPF82-PA11A2) and a DO probe (Endress-Hausser® Oxymax-W COS4).
The control panel was equipped with DO transmitters (Endress-Hausser® Liquisys M COM223) and ORP and pH-temperature transmitters (Endress-Hausser® Liquisys M CPM223). The probes signals were amplified in the transmitters and connected to data acquisition hardware composed of different interface cards (Advantech® PCL-711B, PCL-728 and PCLD-885). These cards also controlled the on/off switch for the mixing device, the peristaltic pumps and the oxygen supply electro-valve. Non-commercial software programmed in LabWindows® was installed in a computer to control the process (Figure 3.2). This software was based on user-friendly interface which allows the creation of the operating cycles, execute them or directly interact with all the devices (mixing, pumps and valves) of the reactor.

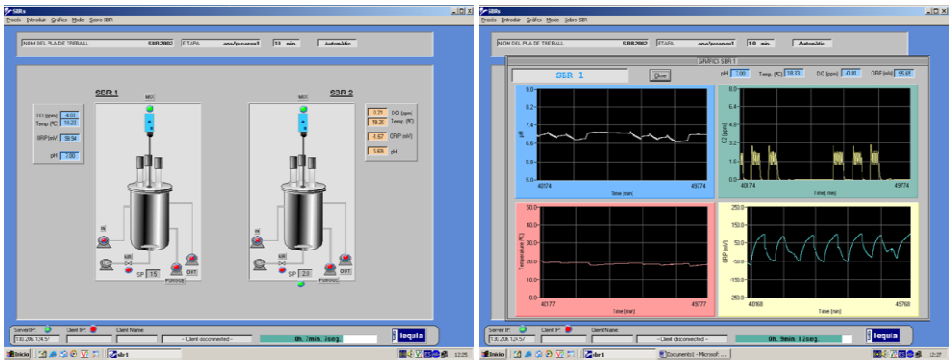

Figure 3.2. Examples of the control interface screen (left) and probes graph interface (right) of the control software.

3.1.2 Batch reactor

The batch reactor (BIOSTAT B-PLUS Sartorius®) was a cylindrical water jacketed reactor with a maximum capacity of 5L. The cap at the top made it possible to operate under sealed conditions and to insert ORP, DO, pH and temperature probes, as well as the stirring device. An integrated control unit contained the gassing system (rotameter, solenoid valves and mass flow controller), peristaltic pumps for pH control and substrate addition and a user-friendly interface. The supervisory software MFCS/DA was used for devices control,

extended visualization, data acquisition and trend display. Figure 3.3 depicts a diagram and an image of the system.

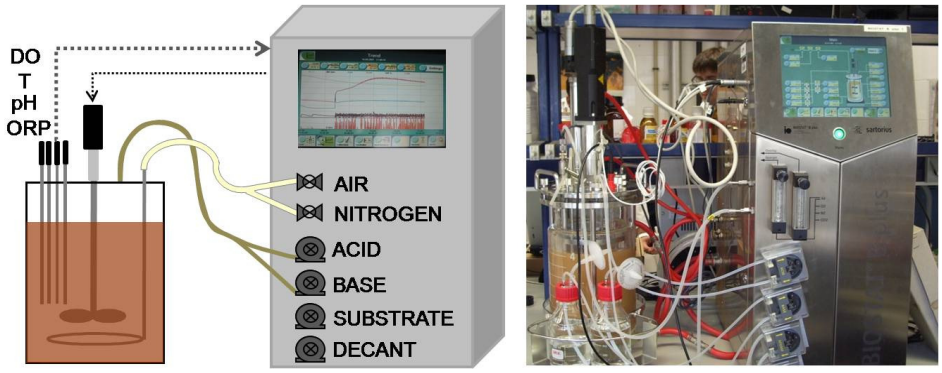

Figure 3.3. Diagram (left) and image (right) of the batch experiments set-up.

3.2 Wastewater

The influent wastewater was stored in a 150L refrigerated tank at 4ºC in order to minimize microbiological activity. The tank was made of stainless steel and equipped with a mixing device so the influent could be introduced in homogeneous conditions.

Synthetic and real wastewater (WW) was used in this thesis. It was prepared in the laboratory and collected from a wastewater treatment plant (WWTP) twice a week to avoid degradation due to long storage.

Synthetic WW was prepared based on Puig *et al*., 2007a as follows (L^{-1}): 0.27 mL ethanol 96%, 560 mg dehydrated meat extract (DME) and 0.4 mL milk as carbon source; 10 mL landfill leachate as a complex organic matter source; 320 mg $NaHCO_3$ as alkalinity source; 180 mg NH_4Cl as ammonium source; 7 mg KH_2PO_4, 18 mg K_2HPO_4 and 14 mg $Na_2HPO_4 \cdot 7H_2O$ as phosphate solution; and 0.19 mg $MnCl_2 \cdot 4H_2O$, 0.0018mg $ZnCl_2 \cdot 2H_2O$, 0.022 mg $CuCl_2 \cdot 2H_2O$, 5.6 mg $MgSO_4 \cdot 7H_2O$, 0.88 mg $FeCl_3 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ as microelements solution. Ethanol only accounted for carbon source while DME, milk and leachate provided both organic matter and nitrogen into the synthetic medium.

Real WW was collected from the Quart WWTP (1000 population equivalent). **Raw wastewater** was stored directly in refrigerated tanks. To obtain decanted WW, the wastewater was retained in a collection tank for 30-60 minutes to separate the particles. This wastewater was considered similar to that obtained from primary settlers. **Decanted WW** was stored in refrigerated tanks for SBR operation. Finally, **decanted WW + EtOH** (ethanol) was obtained by adding 0.12 mL of pure ethanol per liter of decanted WW and storing it in refrigerated tanks for operation.

The average organic matter, the nitrogen and phosphorus composition, and the nutrient ratios of all the influents are summarized in Table 3.1.

Table 3.1. Composition of wastewater used in this thesis.

	Synthetic WW	Particulate WW	Decanted WW	Decanted + EtOH WW	Units
COD_T	593 ± 146	474 ± 21	172 ± 6	281 ± 6	mg COD·L^{-1}
COD_s	452 ± 115	161 ± 8	73 ±6	174 ± 8	mg COD·L^{-1}
BOD_5	529 ± 92	254 ± 3	78 ± 12	183 ± 4	mg BOD·L^{-1}
TN	80 ± 8	83 ± 1	46± 1	45 ± 1	mg N·L^{-1}
NH_4^+	70 ± 6	66 ± 2	38 ± 1	35 ± 1	mg N·L^{-1}
TP	8 ± 1	8 ± 1	5± 2	4 ±1	mg P·L^{-1}
PO_4^{3-}	6.89 ± 1.31	6.17 ± 0.06	3.87 ± 0.02	1.99 ± 0.02	mg P·L^{-1}
TSS	104	166	65	63	mg·L^{-1}
VSS	99	149	65	63	mg·L^{-1}
COD_T/N	8 ± 2	8 ± 3	5 ± 1	10 ± 4	g COD·g^{-1}N
COD_T/P	95 ± 31	101 ± 59	56 ± 24	113 ± 42	g COD·g^{-1}P
COD_T:N:P	100:14:1.2	100:14:1.2	100:23:2.1	100:14:0.9	g COD:g N:g P

3.3 Analyses

Different analytical methods were used throughout this thesis to determine and quantify different parameters in the liquid phase and the solid phase of each system. The biomass was characterized and microbiological analyses were carried out.

3.3.1 Liquid phase

In this section, the methods used for determining the conventional parameters of wastewater are described. The majority are in accordance with *Standard Methods for the Examination of Water and Wastewater* (APHA, 2005). For soluble fraction analysis, the samples were filtered beforehand with a pore size of either 0.45 µm (CODs, N-NH_4^+, TOC, IC) or 0.2 µm (N-NO_2^-, N-NO_3^-, P-PO_4^{3-}) in order to remove suspended solids. As TOC was measured from the soluble fraction it will be referred as dissolved organic carbon (DOC) throughout the thesis. Table 3.2 summarizes the different analytical methods and their references.

Table 3.2. Analytical methods applied in liquid phase.

Analysis		Compound	Observation	Reference
Organic matter	COD	Chemical oxygen demand	Selecta® Bloc digest Crison® Titromatic	APHA-5220C
	BOD_5	Biochemical oxygen demand (at 5 days)	WTW® Oxytop-C	Euro Standard EN 1899-1/1998 APHA-5210B
	TOC	Total organic carbon	Shimadzu® TOC-VC SH	APHA-5310B
Nitrogen	N-TKN	Total Kjeldahl nitrogen	Büchi® Digest system K-437 Büchi® Autokjeldahl Unit K-370	APHA-4500-Norg.B
	N-NH_4^+	Ammonium	Büchi® Autokjeldahl Unit K-370	APHA-4500-NH_3.B-C
	N-NO_2^-	Nitrite	Metrhom® 761-Compact (Metrosep A Supp 5; 250/4.0 mm)	APHA-4110B
	N-NO_3^-	Nitrate	Metrhom® 761-Compact (Metrosep A Supp 5; 250/4.0 mm)	APHA-4110B
	N-TN	Total nitrogen	TN=TKN+NO_2^-+NO_3^-	-
Alkalinity	IC	Inorganic Carbon	Shimadzu TOC-VC SH	APHA-5310B
Phosphorus	P-TP	Total Phosphorus	CECIL Spectrophotometer CE 1021	APHA-4500-P B.5 ; 4500-P E
	P-PO_4^{3-}	Phosphate	Metrhom® 761-Compact (Metrosep A Supp 5; 250/4.0 mm)	APHA-4110B

3.3.2 Solid phase

This section describes the methods to quantify the biomass and analyze the internal polymers of the solid fraction of the system.

3.3.2.1 Total and volatile suspended solids

Solids present in wastewater or inside the reactor (mixed liquor) can be organic or inorganic. Total suspended solids (TSSs) and mixed liquor suspended solids (MLSSs) determine the sum of inorganic and organic fractions of a sample and were analyzed using the 2540D method (APHA, 2005). Volatile suspended solids (VSSs) and mixed liquor volatile suspended solids (MLVSSs) are the organic fraction of the sample and were analyzed according to the 2540E method (APHA, 2005).

3.3.2.2 Internal polymer analysis

Poly-β-hydroxyalkanoates (PHA) are internal polymers present in an enhanced biological phosphorus removal (EBPR) population and used for carbon storage in the cells. As the main PHA, poly-β-hydroxyvalerate (PHV), poly-β-hydroxybutyrate (PHB) and poly-β-hydroxy-2-methylvalerate (PH2MV) were analyzed in accordance to Oehmen (2004). Internal polymer compounds were analyzed in the Chemical Department of the Universidade Nova de Lisboa (UNL, Portugal).

Sample preparation: mixed liquor samples were taken from the reactors and biological activity was stopped by adding two drops of 2% paraformaldehyde solution. Samples were settled and supernatant was decanted. The solid part was freeze-dried for subsequent internal polymer analysis.

Sample digestion: 5 grams of dry sample were digested during 3 hours at 100ºC with 1 mL $CHCl_3$ with heptadecane (internal standard, 10 mg HD/mL) and 1 mL acidic methanol (3% of H_2SO_4 in methanol). Standards were prepared from a stock solution of caproic acid and P(HB-co-HV) pellets (88:12 %, Merck®)

in chloroform. A blank sample (containing neither sample nor standard) was prepared in each batch analyzed.

Sample extraction: 0.5 mL of reverse osmosis (RO) water was added to each sample/standard after it had cooled down. Tubes were stirred for 30 seconds in a vortex and left for phase separation. 800 µL of the chloroform phase (bottom) were extracted and kept in gas chromatography (GC) vials for analysis.

Sample analysis: samples were injected into a DB-Wax (60mx0.53 id) GC column. Pressure was set at 14.5 psi, injector temperature at 280ºC and detector temperature at 250ºC. Final results were calculated taking into account the area and concentration of the internal standard.

3.3.3 Biomass characterization

This section describes different parameters measured to determine the settleability properties and physical characteristics of floccular and granular biomass.

3.3.3.1 Sludge volumetric index

After the suspension was stirred, 1L of mixed liquor is placed in an imhoff cone. The volume occupied by the biomass after n minutes of settling was determined to obtain the settled sludge volume or V_n (n = 1, 5, 10, 15, 20, 25 and 30) (APHA method number 2710C).

The sludge volumetric index (SVI_n) is the volume in milliliters occupied by 1g of suspended solids. The procedure consisted of determining the V_n and divided it by the TSSs in the sample (APHA method number 2710D).

3.3.3.2 Stereomicroscope: size and morphology of the granules

Changes in the morphology of the granules were followed by image analysis (IA) (Jeison and Chamy, 1998). Images of the granular biomass were taken with

a Nikon coolpix-4500 digital camera combined with a stereomicroscope (ZEISS SteREO Discovery V12) using transmitted light (Schott KL2500).

For digital image analysis Quarz PCI software was used, specifically to calculate the mean diameter of the granules. It should be noted that in cases where not enough granules were caught in the pictures analyzed, representativeness might be critical. The mean diameter was calculated as an average of the major axis and the minor axis of the granule (Figure 3.4).

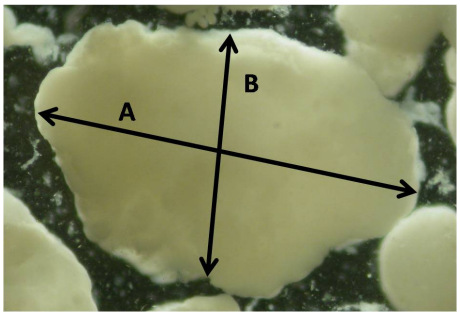

Figure 3.4. Major axis (A) and minor axis (B) of a granule.

Roundness (Equation 3.1), was calculated according to Dulekgurgen *et al.* (2008a). Values of roundness (aspect ratio) were reported between 0 and 1, the former representing a line, and the later a circle or a sphere. The more it diverges from 1, the more the object becomes elliptic.

$$Roundness = \frac{minor\ axis}{major\ axis} \qquad \text{Eq. 3.1}$$

3.3.3.3 Size distribution

The particle size distribution of a granular material dispersed in fluid is a list of values that defines the relative amounts of particles present, sorted according to size. In this thesis, optical counting and laser diffraction were used to obtain the size distribution of mixed liquor samples. The optical counting method was based on IA and the size distribution of the granules was calculated by

reference to a size range and the granules' frequency of occurrence (Figure 3.5A).

To determine size distribution with laser diffraction, a Beckman-Coulter® laser light scattering instrument (LS 13 320 SW) was used. The laser diffraction technique is based on the principle that particles passing through a laser beam will scatter light at an angle that is directly related to their size. This method represents a rapid and robust measurement of particles present in a bulk with a range of 0.02 to 2000 µm (Figure 3.5B).

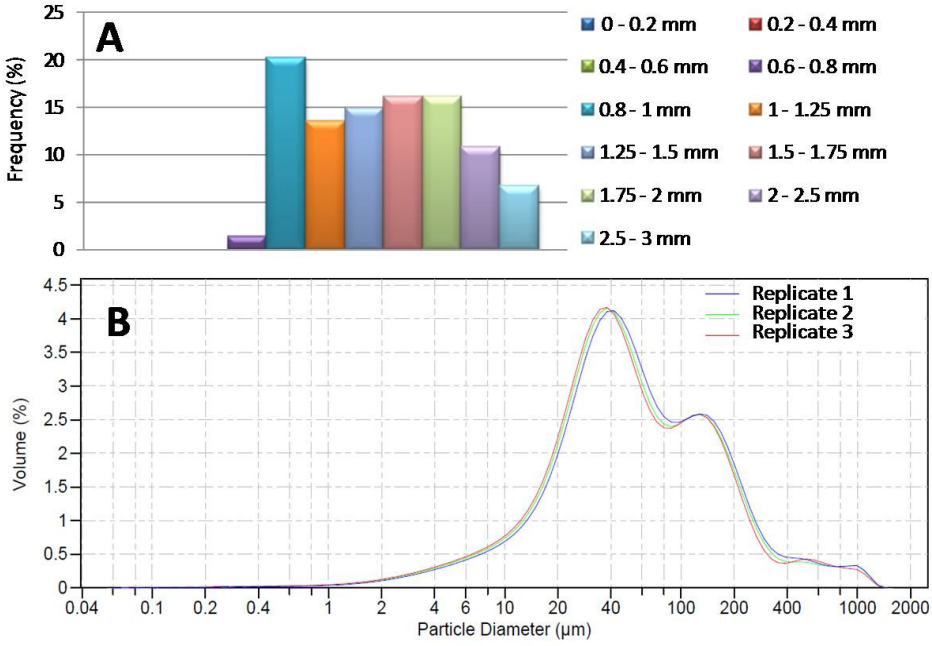

Figure 3.5. Optical counting method (A) and laser diffraction method (B) for size distribution.

3.3.4 Microbiological determination

The biomass morphology and the microbial population composition were studied using high resolution microscopes and molecular techniques.

3.3.4.1 Scanning electron microscope

Morphological studies of the biomass were performed with a ZEISS DSM 960 A (1993) scan electron microscope (SEM) with a magnification capacity ranging from 4 to 300000 and voltage varying from 0.49 to 30 kV. The sludge sample was fixed with glutaraldehyde 2.5% in phosphate buffer for 2 to 4 hours. After fixation, the sample was washed twice with phosphate buffer and then with RO water (3ppb TOC and 18 MΩ). It was then dehydrated using solutions with increasing ethanol concentrations of 40-50%, 60%, 80% and 95% for 10 minutes each time and three times at 100% for 10 minutes. Later, the sample was desiccated by means of critical point dehydration and shaded with gold before it was observed under the SEM.

3.3.4.2 FISH analysis

The identification and abundance of specific microorganisms present in the sludge samples of the reactors was investigated by fluorescence *in situ* hybridization (FISH). In this technique specific regions in 16S rRNA are targeted with fluorescently labeled probes. If the corresponding domain is present, the probe hybridizes to the target sequence and can later be detected microscopically.

FISH analysis in this thesis was performed as specified by Amann (1995) who identified four steps in this molecular technique (Figure 3.6). The first step was the fixation of the sample with 4% paraformaldehyde (PFA) solution for 2-4 hours in order to stop biological activity. Afterwards, samples were washed with phosphate buffer solution (PBS) and kept in PBS:ethanol (1:1) in a freezer for a maximum of 6 months.

The second step was based on the immobilization of the biomass on microscopic slides and the dehydration of the samples by submerging the slides for 3 minutes in ethanol solution with increasing the ethanol concentrations of 50%, 80% and 95%. The third step, hybridization with the selected probes, was carried out under stringent conditions (46ºC, 0-65% formamide) for 1.5 to 2

hours. Speciments were washed with wash buffer at 48ºC. Finally, the target organisms were detected by their characteristic fluorescence.

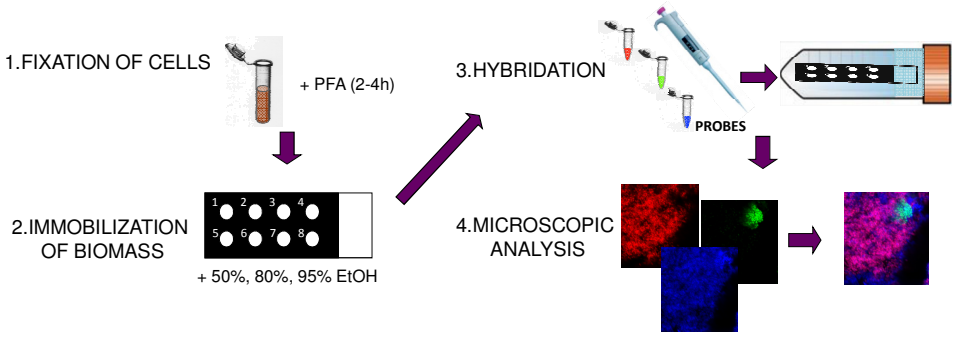

Figure 3.6. General FISH protocol.

The dyes associated with each probe used to detect the specified hybridized rRNA were fluorescein isothiocyanate or FITC (λexc. = 494, λem. = 520) colored green and carbocyanine or CY3 (λexc. = 552, λem. = 568) colored red. To visualize all the cells, samples were hybridized with a mixture of EUB probes with CY5 dye (λexc. = 650, λem. = 670) and colored blue. The probed sludge was examined using a Zeiss® or a Leica® confocal laser scanning microscope (CLSM). The probes used in this thesis are summarized in Table 3.3.

Qualitative or semi-quantitative information of PAO, GAO, AOB and NOB was extracted using mathematical software for image analysis developed in Matlab®. The software was based on the separation of the RGB (red, green, blue) channels and the detection of each positive pixel from a FISH picture. The area containing specific labeled probe cells (FITC or CY3, green or red respectively) was obtained as a percentage of the area of the entire bacterial population (CY5, blue). Quantitative analysis was only considered when more than thirty images per sample were analyzed. In this thesis results were considered in a qualitative point of view because of around ten images per sample were identified. Standard deviation of the mean (standard deviation divided by the square root of the number of images) was evaluated for all cases.

Table 3.3. Oligonucleotids probes used in this thesis.

Name	Specificity	Probe	% Formamide	Reference
EUBMIX	Bacteria domain	EUB338	0-70	Amann *et al.*, 1990
		EUB338-II	0-50	Daims *et al.*, 1999
		EUB338-III	0-50	
PAOMIX	*Accumulibacter*	PAO462	35	Crocetti *et al.*, 2000
		PAO651	35	
		PAO846	35	
GAOMIX	*Competibacter*	GAOQ431	35	Crocetti *et al.*, 2002
		GAOQ989	35	
		GB_G1	35-50	Kong *et al.*, 2002
		GB_G2	35-50	
	Defluviicoccus vanus (cluster 1)	TFO-DF218	35	Wong *et al.*, 2004
		TFO-DF618	35	
	Defluviicoccus vanus (cluster 2)	DEF988	35	Meyer *et al.*, 2006
		DF1020	35	
AOB	Ammonia oxidizing bacteria	NSO190	40	Mobarry *et al.*, 1996
		NSO1225	35	
NOB	Nitrite oxidizing bacteria	NIT3 + compNIT·	40	Wagner *et al.*, 1996
		Ntspa662 + compNtspa662	35	Daims *et al.*, 2000

3.3.4.3 Granule cryostat sectioning

Granules were cut in slices by cryostat sectioning in order to obtain better information of the bacterial distribution. Granules were treated with Tissue-Tek OCT for embedding tissue after fixation and before cryo-sectioning.

The cryo-sectioning embedding protocol aims to remove all traces of salt and ethanol from the sample (previously fixed with PBS:Ethanol solution) and replace it almost completely with OCT. To that aim, samples were washed and placed twice into a 30% sucrose solution at 4ºC for a minimum of two hours. Consecutives washing steps with the same duration and temperature were carried out with OCT:sucrose (30%) at concentrations of 1:3, 1:2, 1:1 and finally

replacing the medium with net OCT. After embedding procedure, granules were transferred into moulds and replenished with OCT.

Granules were sliced in the histology unit in the *Instituto Gulbenkian de Ciência* (Oeiras, Portugal) with a cryostat as the one presented in Figure 3.7. Slices of 10 µm of thickness were placed in superfrost slides and kept in the freezer at -20ºC.

Figure 3.7. Moulds, cryostat and superfrost slides used in the cryo-sectioning protocol.

The later hybridization of granule slices was carried out in the *Instituto de Tecnologia Química e Biologia* (ITQB; Oeiras, Portugal). A viscouse agarose solution was placed surrounding the samples and dried until a barrier to maintain a solution was formed. Afterwards, FISH protocol was applied as specified in section 3.3.4.2.

3.4 Calculations

This section details the results of calculations used in the other chapters, and with which different operational conditions parameters and mass balances were obtained.

3.4.1 SBR operational conditions parameters

Operational conditions parameters of SBRs are sometimes different from the ones used in conventional systems, as they refer to the timing (i.e. cycle definition) and to the working volumes which varies during the operation. Table 3.4 summarizes the main operation parameters used in SBR technology.

Table 3.4. Operating parameters in an SBR (Vives, 2004).

Parameter	Description	Equation	Units
Total cycle time	Sum of the length of all phases: react (t_R), settling (t_S), decant (t_D)	$t_C = t_R + t_S + t_D$	minutes
Reaction time	Sum of filling (t_F), anaerobic (t_A), anoxic (t_{AO}) and aerobic time (t_{AE})	$t_R = t_F + t_A + t_{AO} + t_{AE}$	minutes
Effective fraction	Fraction of the reaction time (i = A, AO or AE) versus to total cycle time	$f_i = 100 \cdot \frac{t_i}{t_C}$	%
Cycles per day	Number of cycles per day	$N_C = \frac{24 \cdot 60}{t_C}$	cycle·d^{-1}
Maximum volume	V_{MIN}: minimum volume V_F: filled volume per cycle	$V_T = V_{MIN} + V_F$	L
Volumetric exchange ratio	Ratio between the fill volume and the maximum reactor volume	$VER = 100 \cdot \frac{V_F}{V_T}$	%
Influent flow	Liters of WW treated per day	$Q_I = V_F \cdot N_C$	L·d^{-1}
Hydraulic retention time	Residence time of a liquid drop inside the reactor	$HRT = \frac{V_T}{Q_I} = \frac{1}{VER \cdot N_C}$	d

3.4.2 Sludge residence time

The sludge age or sludge residence time (SRT) represents the average period of time during which the sludge has remained in the system (Tchobanoglous *et al.*, 2003). SRT in this thesis has been calculated in accordance with Seviour and Blackall (1999) and is defined in Equation 3.2.

$$SRT = \frac{Volume\ of\ liquid\ in\ the\ reactor \cdot Sludge\ MLSS}{(Sludge\ wastage\ rate \cdot MLSS) + (Effluent\ discharge\ rate \cdot Effluent\ TSS)} \quad \text{Eq. 3.2}$$

3.4.2.1 Aerobic sludge residence time

SBR technology is based on different conditions being applied in the same reactor. When the treatment process involves alternating anaerobic, aerobic and anoxic conditions, the SRT calculated will express the sludge age of all microbial cells in the system. In order to calculate the specific sludge age for every condition, the anaerobic, anoxic or aerobic contribution in the cycle should be taken into account. Equation 3.3 defines how aerobic SRT (SRT_{AER}) has been calculated in this thesis.

$$SRT_{AER} = SRT \cdot \frac{\%\ AER}{100} = SRT \cdot \left(\frac{aerobic\ time}{cycle\ time}\right) \qquad \text{Eq. 3.3}$$

3.4.2.2 Minimum aerobic SRT

The design for SRT nitrification must be selected with caution as variable nitrification growth rates have been observed at different sites. Nitrification is temperature-dependent, but temperature has a differential effect on AOB and NOB activity. To address this issue, Figure 3.8 shows the minimum aerobic SRT ($minSRT_{AER}$) needed for the development of both communities as a function of temperature.

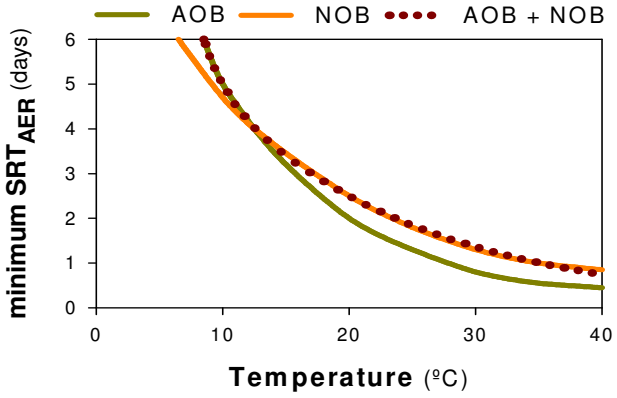

Figure 3.8. Minimum aerobic SRT for AOB, NOB and AOB + NOB as a function of temperature (adapted from Hellinga *et al.*, 1998).

Equation 3.4 defines the fit curve obtained from the SRT-temperature graph (red dotted line, Figure 3.8) and can be used to calculate the minimum aerobic SRT for complete nitrification.

$$minSRT_{AER}\,(T) = 122 \cdot e^{-0.56T} + 8.36 \cdot e^{-0.06T} \qquad \text{Eq. 3.4}$$

3.4.3 Free nitrous acid

The concentration of free nitrous acid (FNA) in mg N-$HNO_2 \cdot L^{-1}$ was calculated as a function of pH, temperature (T) in Celsius degrees and total nitrite (TNO_2) in mg N-$NO_2^- \cdot L^{-1}$ as shown in Equations 3.5 and 3.6 (Anthonisen *et al.*, 1976).

$$K_{e,HNO_2} = e^{\frac{-2300}{273+T}} \qquad \text{Eq. 3.5}$$

$$FNA = \frac{TNO_2}{1+\left(\frac{K_{e,HNO_2}}{10^{-pH}}\right)} \qquad \text{Eq. 3.6}$$

3.4.4 Simultaneous nitrification-denitrification efficiency and mass balance

Simultaneous nitrification-denitrification (SND) can take place as a result of an existing oxygen gradient, with denitrification occurring in the areas of low dissolved oxygen (DO) concentrations (Seviour and Blackall, 1999). SND efficiency was calculated taking into account the nitrogen denitrified under aerobic conditions (Zeng *et al.*, 2003c; Equation 3.7). Values obtained were considered as maximum SND because ammonium assimilation by heterotrophic bacteria was not taking into account.

$$\%\,SND = \frac{denitrification}{nitrification} = \frac{[NH_4^+]_0 + [NO_3^-]_0 + [NO_2^-]_0 - [NH_4^+]_{END} - [NO_3^-]_{END} - [NO_2^-]_{END}}{[NH_4^+]_0 - [NH_4^+]_{END}} \qquad \text{Eq. 3.7}$$

SND was usually easily observed when analyzing cycle studies due to an unbalanced nitrite or nitrate production compared with ammonium oxidation. However, SND was more difficult to detect when evaluating effluent concentrations. For this reason, experimental values (Equation 3.8) could be compared with theoretical NO_x^- values (the sum of nitrite and nitrate concentrations) in the effluent (Equation 3.9) to determine the state of the system in terms of denitrification performance. Only the second feeding event of the cycle (accounting for 30% of the total feed volume, V_w) was taken into account for the theoretical concentration.

$$Experimental\ NO_x^- = [NO_2^-]_{EFFL} + [NO_3^-]_{EFFL} \qquad \text{Eq. 3.8}$$

$$Theoretical\ NO_x^- = ([NH_4^+]_0 - [NH_4^+]_{EFFL}) \cdot \frac{0.3 \cdot V_W}{V_{max}} \qquad \text{Eq. 3.9}$$

Denitrification performance could vary during a cycle and this could be detected by the differences between experimental and theoretical values. Figure 3.9 depicts the three main cases that could occur in the reactor in terms of denitrification performance.

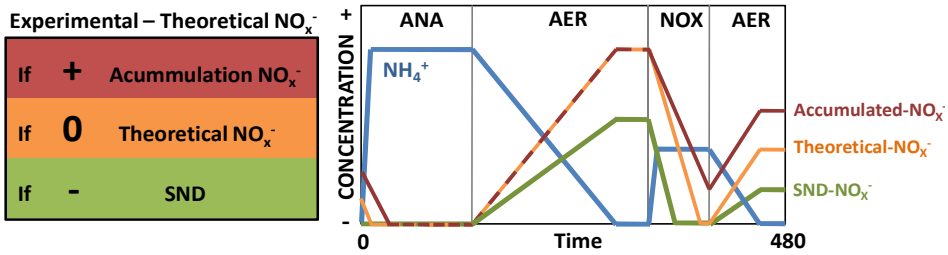

Figure 3.9. Threshold values (left) and ammonium (NH_4^+) and nitrate and nitrite (NO_x^-) theoretical profiles for different denitrification scenarios.

Accumulation of NO_x^- occured when organic matter or the anoxic phase length was insufficient during denitrification. In such a case the experimental concentration was higher than the theoretical one and the difference was negative. When the experimental performance behaved in the same way as the theoretical, the values of the difference were zero. Finally, a lower concentration of nitrate and/or nitrite in the effluent indicated unbalanced

nitrogen production compared with ammonium oxidation. Therefore, positive values for the experimental and theoretical difference implied the occurrence of SND under aerobic conditions.

RESULTS

Chapter 4. SBR conditions for long-term operation in a conventional nutrient removal system

This chapter is focused on the reestablishment of biological nutrient removal after an increase of the organic, nitrogen and phosphorus loading rates applied in a floccular reactor. The study provides guidance on how nitrification, denitrification and enhanced biological phosphorus removal process can be recovered on a sequencing batch reactor. Nitrogen and phosphorus removal cannot be recovered by applying the same operational conditions. First, nitrification should be recovered by modifying aerobic phases. Later, feed strategy and anaerobic phase length should be adjusted for denitrification and phosphorus removal. The final operation was able to work for long-term operation at a volume exchange ratio of 40%, leading to a bigger loading treatment. All objectives have been approached from chemical, engineering and microbiological points of view.

This chapter is based on the following publication:

Coma, M., Puig, S., Monclús, H., Balaguer, M.D., Colprim, J. (2010). Effect of cycle changes on simultaneous biological nutrient removal in a sequencing batch reactor (SBR). *Environ. Technol*. **31** (3): 285-294.

4.1 Introduction

Biological nutrient removal (BNR) has been widely studied and applied during the last few decades (Grady and Lim, 1980; Tchobanoglous *et al*., 2003, see section 1.2.3 and 1.2.4). Continuing high demand for water and the effects of anthropogenic uses have forced wastewater treatment plants (WWTPs) to operate at their maximum capacity. An increase in load can often lead to the destabilization of the microbial population in the system and, as a consequence, the breakdown of the nutrient removal process (Hu *et al.*, 2010). When the disturbance of these reactions occur in the same basin, as in a sequencing batch reactor (SBR), the stability of the system is sometimes hard to manage because different conditions are required to perform nutrient (nitrogen and phosphorus) removal. Working with both processes simultaneously requires a compromise in operational conditions. Hence, when the performance is destabilized, consecutive steps focusing on each individual nutrient have to be carried out before all the reactions are coupled together.

4.2 Objectives

This chapter focused on the recovery of nitrogen and phosphorus removal after a load increase. The objective was to determine which strategy, such as feed pattern or phase length or distribution, should be applied to reestablish nutrient removal. Later on, the study concentrated on the optimization of the long-term operation and application of high carbon, nitrogen and phosphorus loads when treating sewage streams.

4.3 Experimental procedure

A 30L lab-scale SBR (see section 3.1.1) fed with synthetic wastewater (see section 3.2) with average COD, nitrogen and phosphate concentrations of 568 ± 134 mg COD·L^{-1}, 81 ± 7 mg N-TKN·L^{-1} and 7 ± 1 mg P-PO_4^{3-}·L^{-1} respectively, was used in this study. The SBR was seeded with activated sludge from a BNR WWTP and loaded with 0.41 Kg COD·$m^{-3}d^{-1}$, 61 g N·$m^{-3}d^{-1}$ and 4.7 g P·$m^{-3}d^{-1}$

before the experimental study took place, achieving removal efficiencies of 91% for nitrogen and 99% for phosphorus removal.

To simulate a load increase, the volume exchange ratio (VER) was raised from 28% to 40% by increasing the loading rates to 0.62 Kg COD·$m^{-3}d^{-1}$, 95 g N·$m^{-3}d^{-1}$ and 7.8 g P·$m^{-3}d^{-1}$. The daily flow of the SBR was raised to 33 L·d^{-1}, thereby reducing the value of the hydraulic retention time (HRT) from 1.24 to 0.83 days.

The SBR worked in 8h cycles beginning with an anaerobic-aerobic pair of phases focusing on enhanced biological phosphorus removal (EBPR), followed by other sequences of anoxic-aerobic phases for nitrogen removal, and finishing with settling and drawing phases of 39 and 15 minutes, respectively. The sludge retention time (SRT) was maintained at around 21 days by wastage at the end of the reaction time. The dissolved oxygen (DO) set-point was fixed at 1.5 mg O_2·L^{-1}. Wastewater was always introduced under anaerobic or anoxic conditions in order to enhance phosphate release and denitrification, using the easily biodegradable organic matter from the wastewater (Puig *et al.*, 2007b). Figure 4.1 shows the two different cycles applied during the study. The step-feed strategy (three feeding steps, Figure 4.1A; two feeding steps, Figure 4.1B) was modified according to the system status, in terms of the number of feedings events, the volume treated in each feeding phase and the length of each phase.

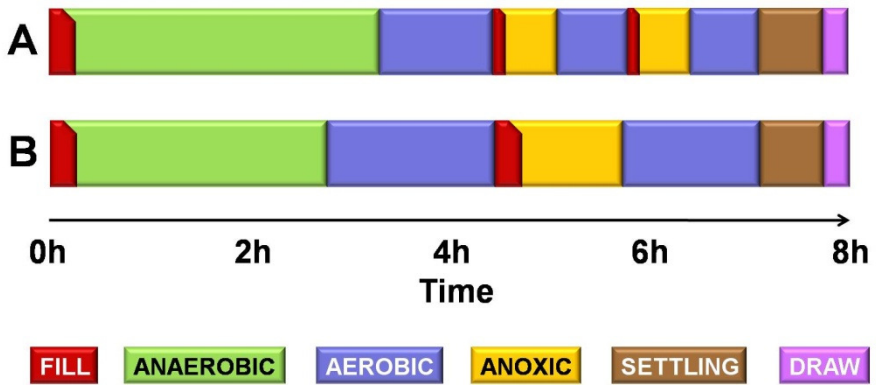

Figure 4.1. Working cycles applied in the study. A) three feeding events; B) two feeding events.

4.4 Results

An SBR working for nitrogen and phosphorus removal treated synthetic wastewater and demonstrated efficiencies for both nutrients of over 91%. At day 0 of the study, the load was increased from 0.41 Kg COD·$m^{-3}d^{-1}$, 61 g N·$m^{-3}d^{-1}$ and 4.7 g P·$m^{-3}d^{-1}$ to mean values of 0.62 Kg COD·$m^{-3}d^{-1}$, 95 g N·$m^{-3}d^{-1}$ and 7.8 g P·$m^{-3}d^{-1}$ for organic, nitrogen and phosphorus loading rates, respectively. This change was applied in order to study the effect on BNR performance when higher volumes of wastewater have to be treated, for example when there is a population increase in summer periods. Figure 4.2 depicts the organic loading rate (OLR), nitrogen loading rate (NLR) and phosphorus loading rate (PLR) over the whole study.

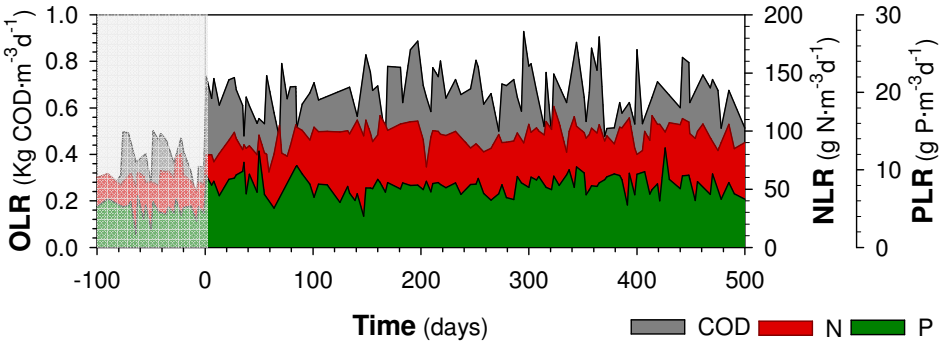

Figure 4.2. Organic loading rate (OLR), nitrogen loading rate (NLR) and phosphorus loading rate (PLR) applied over the study period. Shadowed zone corresponds to previous operation at lower loads.

As can be seen in Figure 4.2, the OLR had higher variability than nitrogen and phosphorus, ranging from 0.47 to 0.88 Kg COD·$m^{-3}d^{-1}$ due to organic matter degradation in the storage tank, even though the wastewater was kept at 4ºC.

After the loading shock at day zero, the system was kept working with the same cycle (Figure 4.1A) and conditions which had previously operated under from day -100 to 0. Figure 4.3 presents the main organic matter, nitrogen and phosphorus removal efficiencies obtained throughout the whole of the study.

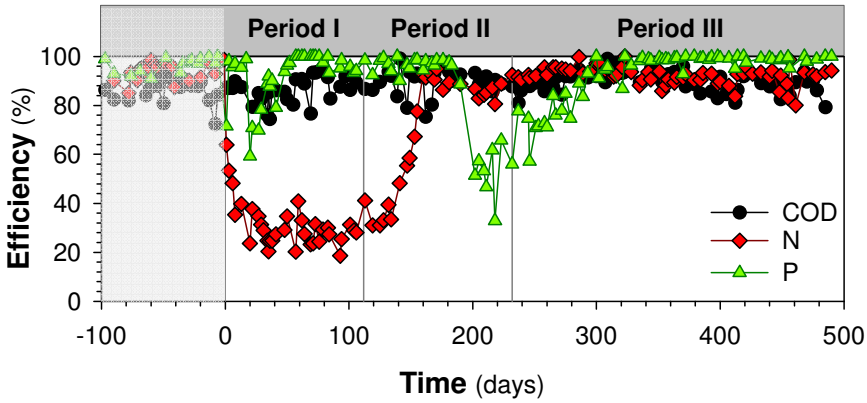

Figure 4.3. Efficiency of organic matter (COD), nitrogen (N) and phosphorus (P) removal over the study period. Shadowed zone corresponds to previous operation at lower loads.

Disturbances in the nutrient removal process were seen as soon as the VER, and as a consequence the OLR, were increased from 28% to 40% at day zero. The results of the study have been divided into three periods according to the strategies applied to recover the BNR's performance. Table 4.1 summarizes the operational conditions for each period.

Table 4.1. Operational conditions applied for biological nutrient removal during the study.

Period	Range SRT Days	SRT_{AER} Days	Strategy Figure 4.1	Feeding Volumes Ana	Feeding Volumes Anx	Cycle distribution* Ana	Cycle distribution* Anx	Cycle distribution* Aer
I	4-13	1.2 - 4	A	50%	25%-25%	49%	20%	31%
II	16-25	7 - 11	A/B	50%	50%	38%	20%	42%
III	10-22	4 - 7	B	70%	30%	38%-48%	20%	42%-32%

*Anaerobic (Ana), anoxic (Anx) and aerobic (Aer) percentages are calculated over the reaction time.

4.4.1 Period I. BNR destabilization and spontaneous granular formation

On day 0 of the study, the SBR was running for BNR purposes on the basis of a three-feeding events step-feed strategy (Figure 4.1A). This configuration had been successfully applied when the working VER was 28%. However, when the VER was increased to 40% on day 0, a destabilization of the performance was observed. To study the causes of this disturbance in depth, different parameters were evaluated. Figure 4.4 shows the mixed liquor suspended solids (MLSS), nitrogen compounds and phosphorus concentrations in the effluent of the SBR during Period I.

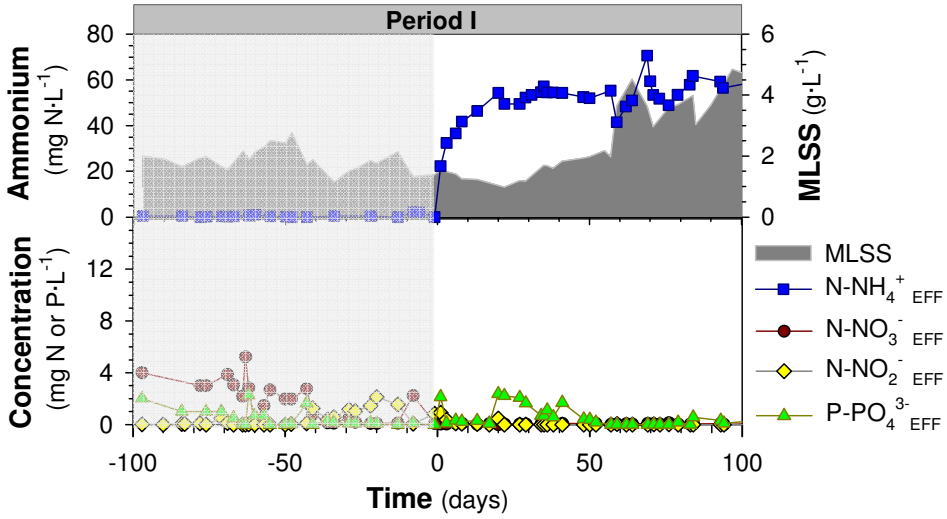

Figure 4.4. Ammonium ($NH_4^+{}_{EFF}$) effluent concentrations and MLSS (top) and nitrate ($NO_3^-{}_{EFF}$), nitrite ($NO_2^-{}_{EFF}$) and phosphate ($PO_4^{3-}{}_{EFF}$) effluent concentrations (bottom) during the Period I. Shadowed zone corresponds to previous operation at lower loads.

The MLSS presented in Figure 4.4 (top) show a smooth decrease from 1.6 to 1.1 g MLSS·L^{-1} until day 30 due to a biomass washout caused by a bulking phenomenon. The solids wastage in the effluent caused an SRT decrease from 21 days (from the previous operation) to 3 days. From that moment on, automatic wastage was stopped in order to recover the biomass. However, around 300 mg TSS·L^{-1} were discharged through the effluent for the rest of the period, with filamentous bacteria being removed from the system and the

bigger particles selected to stay in. From day 50, compacted particles of biomass, defined as granules, appeared in the reactor and the MLSS increased to 4.8 $g \cdot L^{-1}$. Even though the biomass concentration in the system was increased, the SRT only went up to 13 days due to effluent wastage.

In terms of nutrient removal, ammonium in the effluent rose to 54.3 mg $N\text{-}NH_4^+ \cdot L^{-1}$, obtaining a nitrification efficiency about 30%, and remained at around this concentration until the end of Period I. Ammonium oxidizing bacteria (AOB) was probably washed out due to low values of SRT between 3 and 13 days (Table 4.1). According to Tchobanoglous *et al.* (2003), the minimum aerobic SRT (see section 3.4.2.1) that will allow AOB growth is around 5-7 days at 20ºC. The SRT_{AER} presented during Period I was between 1.2 and 4 days, not enough for nitrifiers to grow. The system was therefore not able to restore nitrification capacity after the loading increase at day 0, not even when the biomass concentration was increased and remained stable from day 60 at around 4.0 g $TSS \cdot L^{-1}$.

With regards to phosphate concentration (Figure 4.4, bottom), the effluent remained below the limited discharge level (2.00 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$), except between days 20 and 30 when the phosphate effluent concentration increased from 0.07 to 2.36 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$, which coincided with the minimum solids concentration. At day 38 of the study, a cycle profile analysis was carried out to measure the EBPR. Figure 4.5 shows the phosphate, nitrate and nitrite concentrations during the SBR's first anaerobic and aerobic phases.

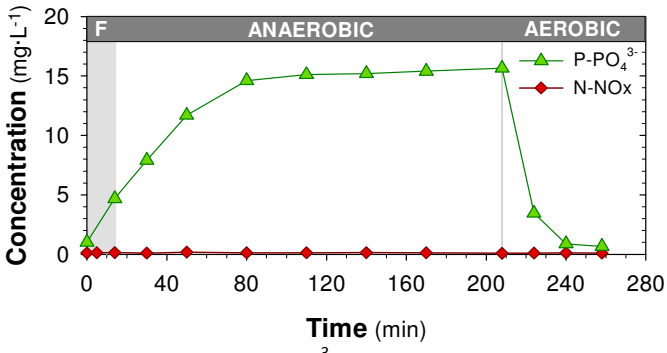

Figure 4.5. Evolution of phosphate (PO_4^{3-}), nitrate and nitrite (NOx) concentrations during the first anaerobic-aerobic phase of the cycle at day 38. Shadowed zone corresponds to the filling phase.

The profile during the anaerobic phase showed a stabilization of phosphate release at 15 mg $P\text{-}PO_4^{3-}\cdot L^{-1}$ in 80 minutes, which accounts for 38% of the total anaerobic time (3.5 mg $P\cdot g^{-1}VSS\cdot h^{-1}$ of phosphate release rate). Phosphate was taken up in 30 minutes with a phosphate uptake rate (PUR) of 11.4 mg $P\text{-}PO_4^{3-}\cdot g\ VSS^{-1}\cdot h^{-1}$ under aerobic conditions. Neither nitrate nor nitrite appeared during the aerobic phase, due to a lack of nitrification. At the end of Period I, therefore, organic matter and phosphorus removal (Figure 4.3) achieved efficiencies of 89% and 98%, respectively, while nitrogen remained at 30% efficiency limited by the nitrification process.

4.4.2 Period II: cycle adaptations for nitrogen removal

Period I ended with the appearance of granular sludge due to biomass selection through effluent wastage. This increased the biomass concentration in the reactor, but did not help nitrification recovery, with over 50 mg $N\text{-}NH_4^+\cdot L^{-1}$ being discharged at the end of the cycle. The cause, as previously noted, was the low SRT_{AER} which did not allow AOB development. EBPR achieved big removals of around 98%. Phosphorus release stabilization was reached in less than 40% of the anaerobic phase. In order to improve nitrifying bacteria growth, the SBR cycle could be modified by increasing the aerobic time to the detriment of the anaerobic reaction time. The aerobic time was therefore increased from 31% to 42% of the total reaction time (Table 4.1) on day 112 of the study at the beginning of Period II. Figure 4.6 shows the mixed liquor suspended solids (g $MLSS\cdot L^{-1}$), nitrogen compounds (mg $N\cdot L^{-1}$) and phosphorus (mg $P\cdot L^{-1}$) concentrations in the effluent during Period II.

The SBR was run for 23 days with the new cycle while applying longer aerobic conditions (42% instead of the previous 31%, Table 4.1). During this time the SRT_{AER} was increased from 4.0 to 10.4 days. Even with optimal aerobic conditions for AOB growth, there was a high level of ammonium concentration remaining in the effluent (53.2 ± 2.4 mg $N\text{-}NH_4^+\cdot L^{-1}$). The problem could have been explained as a phenomenon of transient response for maximum activity in changing conditions (from the anoxic to the aerobic phases). Vanrolleghem *et al.* (2004) observed that nitrifiers, similar to the heterotrophic bacteria, reach the maximum activity after a transient response. A prolonged transient is observed with nitrifiers subjected to famine conditions. In an attempt to

enhance the nitrification process, the cycle was modified at day 135 from three to two feeding events, with the last two anoxic-aerobic phases being joined together to treat the remaining 50% of wastewater per cycle (Figure 4.1B, Table 4.1). This allowed lengthening the aerobic phase and avoiding changing conditions which caused longer transient response periods. The SRT_{AER} obtained after joining the aerobic phases was the same as presented before but avoiding the transient response time. As can be seen in Figure 4.6 (top), after applying this change (day 135), the ammonium concentration in the effluent decreased from 55.5 to 1.1 mg $N\text{-}NH_4^+ \cdot L^{-1}$ in less than four weeks. Nitrogen removal was kept under the discharge limit (15.0 mg $N \cdot L^{-1}$) for the rest of the study.

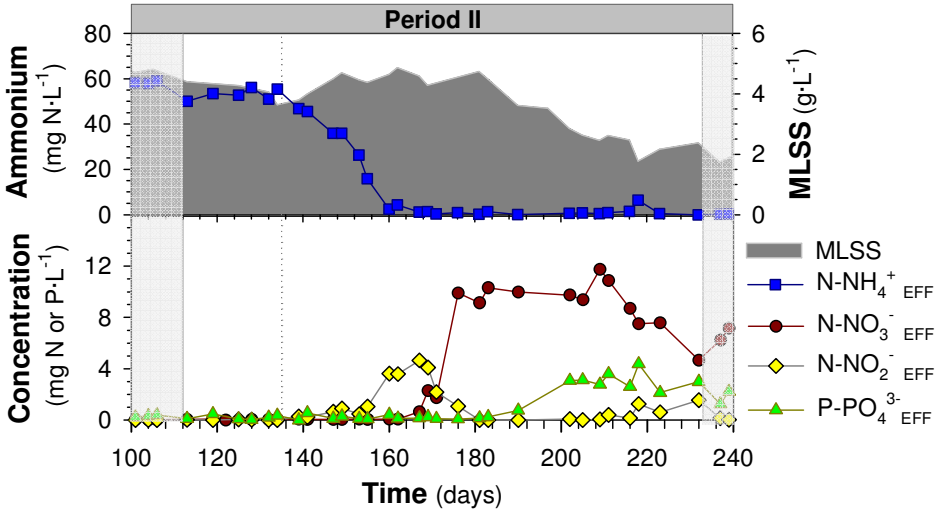

Figure 4.6. Ammonium ($NH_4^+{}_{EFF}$) effluent concentrations and MLSS (top) and nitrate ($NO_3^-{}_{EFF}$), nitrite ($NO_2^-{}_{EFF}$) and phosphate ($PO_4^{3-}{}_{EFF}$) effluent concentrations (bottom) during Period II. Shadowed area corresponds to Period I and III.

Once the nitrification process had been completely recovered on day 160, up to 5 mg $N\text{-}NO_2^- \cdot L^{-1}$ were observed in the effluent (Figure 4.6, bottom). This was due to the decoupling of nitrifying bacteria, as nitrite oxidizing bacteria (NOB) require the nitrite produced by AOB as substrate in order to grow. When NOB were developed, nitrite disappeared while nitrate gradually increased, reaching values of up to 10.3 mg $N\text{-}NO_3^- \cdot L^{-1}$ on day 183 (Figure 4.6, bottom). Figure 4.7

presents ammonium, nitrate, nitrite and phosphate profiles from aerobic phases during the period where NOB developed (day 161 and day 169).

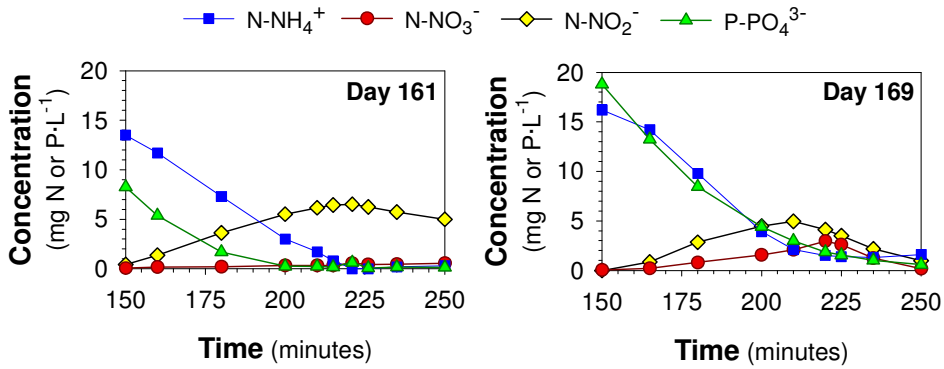

Figure 4.7. Ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) profiles during the aerobic phase from day 161 (left) and day 169 (right) of the study.

During the first aerobic profile at day 161 (Figure 4.7, left), 13.5 mg $N\text{-}NH_4^+ \cdot L^{-1}$ were completely oxidized in around 75 minutes. Nitrite was the sole product of nitrification, reaching a concentration of 5 mg $N\text{-}NO_2^- \cdot L^{-1}$ at the end of the aerobic phase, which coincided with the maximum nitrite concentration achieved in the effluent during Period II (Figure 4.6). Some days later, at day 169, NOB started to develop. 16.2 mg $N\text{-}NH_4^+ \cdot L^{-1}$ were removed and a maximum of 5 mg $N\text{-}NO_2^- \cdot L^{-1}$ and 2.6 mg $N\text{-}NO_3^- \cdot L^{-1}$ were obtained during the aerobic period (Figure 4.7, right). Furthermore, at the end of the phase virtually no nitrite or nitrate were detected. The differences presented between the nitrogen oxidized and the nitrite and nitrate detected at the end of the aerobic phase showed that 61% and 93% of simultaneous nitrification-denitrification (SND, see section 1.2.5 and 3.4.4) occurred at days 161 and 169, respectively. Aerobic conditions were maintained at 1.5 mg $O_2 \cdot L^{-1}$, so SND was probably enhanced by substrate and oxygen diffusion through the granules present in the system.

In terms of phosphorus performance (Figure 4.7), both aerobic phases removed all phosphate released in the previous anaerobic phase. Phosphate uptake rates (PURs) were 2.4 and 3.2 mg $P\text{-}PO_4^{3-} \cdot g^{-1}$ $VSSh^{-1}$ at days 161 and 169, respectively. These values were far lower than the PUR from Period I (11.4 mg $P\text{-}PO_4^{3-} \cdot g^{-1}$ $VSSh^{-1}$). Various hypotheses to explain this reduction were examined.

Firstly, the remaining nitrite in the effluent would affect EBPR in the subsequent anaerobic phase because of organic matter competition between PAOs and heterotrophic denitrifiers (van Loosdrecht *et al*., 1997b). Secondly, aggregated biomass would enhance simultaneous denitrification and phosphorus removal by denitrifying PAOs (DPAOs) which presents lower rates than totally aerobic EBPR (Tsuneda *et al*., 2006). Finally, a nitrite threshold of 2 mg $N\text{-}NO_2^-\cdot g^{-1}$ VSS, or for the free nitrous acid (FNA) of over 0.002 mg $N\text{-}HNO_2\cdot L^{-1}$ as a consequence of nitrite presence, has been reported as an inhibitor for phosphorus uptake with nitrite non-acclimated sludge (Saito *et al*., 2004; Zhou *et al*., 2007; Pijuan *et al*., 2010). In contrast, the maximum theoretical FNA achieved when phosphorus was being uptaken was calculated as 0.012 mg $N\text{-}HNO_2\cdot L^{-1}$ (at 15ºC and a minimum pH of 6.6) (see section 3.4.3). In order to evaluate the inhibitory effect of FNA on phosphorus removal, experimental and theoretical data were assessed. The theoretical expression was adjusted to the experimental results using the Monod kinetic term shown in Equation 4.1. This equation was chosen because it was the one presenting a better prediction of the FNA inhibitory effect according to Zhou *et al.* 2007.

$$PUR = PURmax\ \frac{S_{HNO_2}}{K_S + S_{HNO_2}}\ e^{-\alpha S_{HNO_2}} \quad \text{where} \quad \alpha = \frac{1}{S^*_{HNO_2}} \qquad \text{Eq. 4.1}$$

where PUR is the phosphate uptake rate (mg $P\cdot g^{-1}VSSh^{-1}$) and S_{HNO_2} is the FNA concentration (mg $N\text{-}HNO_2\cdot L^{-1}$).

The estimated maximum PUR (PUR*max*) was 6.7 mg $P\cdot g^{-1}VSSh^{-1}$ and the aparent affinity constant (K*s*) was $2.2\cdot10^{-4}$ mg $N\text{-}HNO_2\cdot L^{-1}$. A specific inhibition constant (α) was dependent of $S^*_{HNO_2}$, which represents the FNA concentration where PUR is a 37% (e^{-1}) of the PUR*max*. The value found for α was 111.9 $L\cdot mg^{-1}$ $N\text{-}HNO_2$. Figure 4.8 shows the correlation of experimental and theoretical results between P-uptake rate and FNA concentration. The adjusted curve presented an r^2 of 0.89.

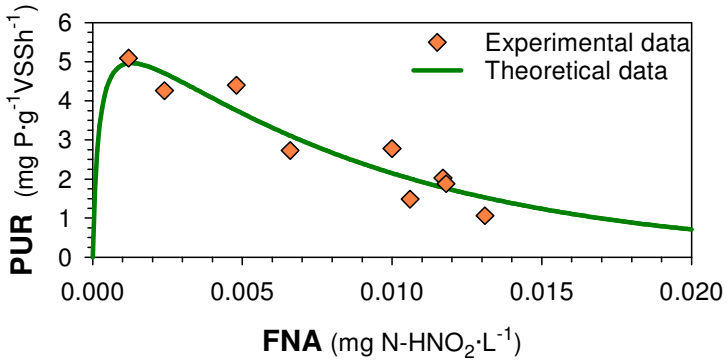

Figure 4.8. Correlation between P-uptake rate (PUR) and free nitrous acid concentration (FNA). Solid line is the prediction of Equation 4.1 from Zhou *et al.* (2007).

The data showed a reduction in the PUR when the FNA concentration was increased. However, the apparent affinity constant obtained had a higher value ($2.2 \cdot 10^{-4}$ mg $N\text{-}HNO_2 \cdot L^{-1}$) than the values from the literature ($3.1 \cdot 10^{-5}$ mg $N\text{-}HNO_2 \cdot L^{-1}$; Zhou *et al.* 2007). This could be due to the presence of granular sludge, as the FNA concentration from the media is reduced inside the granules by substrate diffusion. Because of that, the aparent affinity constant would present higher values for granular sludge rather than floccular sludge.

Therefore, the presence of FNA reduced the PUR compared with the rates measured in Period I (2.4 mg $P\text{-}PO_4^{3-} \cdot g^{-1}$ $VSSh^{-1}$ and 11.4 mg $P\text{-}PO_4^{3-} \cdot g^{-1}$ $VSSh^{-1}$, respectively), even though phosphorus concentration in the effluent remained at low values (< 0.6 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$), probably because of DPAO activity. However, when NOB grew, nitrate reached its maximum concentration of 10 mg $N\text{-}NO_3^- \cdot L^{-1}$ at around day 190. As a consequence, a mean concentration of 3.09 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$ remained in the effluent (Figure 4.6, bottom). This was due to the double effect of EBPR deterioration in the presence of FNA, as nitrite still appeared as an intermediate under aerobic conditions (Figure 4.7, right), and organic matter competition for denitrification and phosphorus removal.

The cycle modifications applied in Period II restored the nitrification process. The increase in the aerobic percentage from 31% to 42%, together with the reduction in alternating conditions by the application of two feed events instead of three, provided a successful solution. However, the nitrite and

nitrate produced by nitrifiers when nitrification efficiencies reached values of 98% interfered with EBPR performance and reduced phosphorus removal (Figure 4.3). At the end of Period II BNR efficiencies were 85% and 57% for nitrogen and phosphorus removal, respectively.

4.4.3 Period III: cycle optimization for nutrient removal

The presence of nitrite and nitrate in the SBR adversely affected EBPR performance because of FNA inhibition and organic matter competition between PAOs and heterotrophic denitrifiers in the subsequent anaerobic phase. Therefore, EBPR removal efficiencies decreased from 96% to 57%.

At the end of Period II the SBR was operating with a step-feed strategy with two feeding events (Figure 4.1B). The first feed (50% of the water to treat) was introduced under anaerobic conditions for EBPR purposes. The second feed (50%) was used to denitrify nitrate produced by nitrification from the previous aerobic phase. In order to enhance PAO growth and avoid organic matter limitation during the anaerobic phases, the volume of wastewater introduced at the beginning of the cycle was increased at expense of the second feed. However, the minimum volume required in the second feed had to be enough to denitrify the nitrogen oxidized and to meet the requirements at the end of the cycle. For this reason, and in order to get the most suitable feed distribution, COD and nitrogen mass balances were carried out, taking into account the mean organic matter from the influent (548 mg COD·L^{-1}), the mean ammonium concentration (69 mg N-NH_4^+·L^{-1}), the volume feed per cycle (11L) and the minimum volume of the reactor (16L). Table 4.2 summarizes the different scenarios considered. **Nox** stands for nitrogen oxidized in the first aerobic phase, **COD req** for organic matter required for denitrification (2.86 g COD·g N-NO_3^-, Tchobanoglous *et al.*, 2003) in the anoxic phase, **COD avail** for organic matter available for denitrification and **N eff** for nitrogen remaining in the effluent after the last aerobic phase.

Table 4.2. COD and nitrogen mass balances to determine suitable feed distribution over the cycle.

Scenario	Feed 1 %	Feed 2 %	N ox mg N·L^{-1}	COD req mg COD·L^{-1}	COD avail mg COD·L^{-1}	N eff mg N·L^{-1}
1	50	50	17.7	50	112	14.1
2	55	45	18.9	54	100	12.7
3	60	40	20.2	58	89	11.2
4	65	35	21.3	61	78	9.8
5	70	30	22.4	64	67	8.4
6	75	25	23.5	67	56	7.0

As shown in Table 4.2, a distribution of 70% and 30% (Scenario 5) in the first and second feeds, respectively, was the best option in terms of obtaining the minimum nitrogen in the effluent. Denitrification of all the nitrogen introduced on the first feed with the available organic matter was considered. If there was less volume percentage in the second feed (Scenario 6), it would end up with no available organic matter for denitrification (e.g. 56 mg COD·L^{-1} available as against 67 mg COD·L^{-1} required), although nitrate concentration in the effluent would be lower because of a dilution effect. Besides, it can be observed that theoretical values presented in Table 4.2 are higher than experimental results (mean values of 10 mg N-NO_3^- when 50%-50% feed pattern was applied, Figure 4.6). This fact was due to SND presented in aerobic cycles (Figure 4.7), thus experimental values would be even lower than the theoretical results.

Finally, the volume of wastewater introduced in each cycle was changed to a distribution of 70% and 30% in the first and second feeds, respectively (Table 4.1) at the beginning of Period III. The length of phases was kept as in Period II (Figure 4.1B). The new filling configuration resulted in a higher organic matter load in the first anaerobic phase of the cycle (178 mg COD·L^{-1} as against of 112 mg COD ·L^{-1} from the previous operation), while the second part was enough to ensure nutrient removal. Figure 4.9 shows the mixed liquor suspended solids (g MLSS·L^{-1}), nitrogen compounds (mg N·L^{-1}) and phosphorus (mg P·L^{-1}) concentrations in the effluent during Period III.

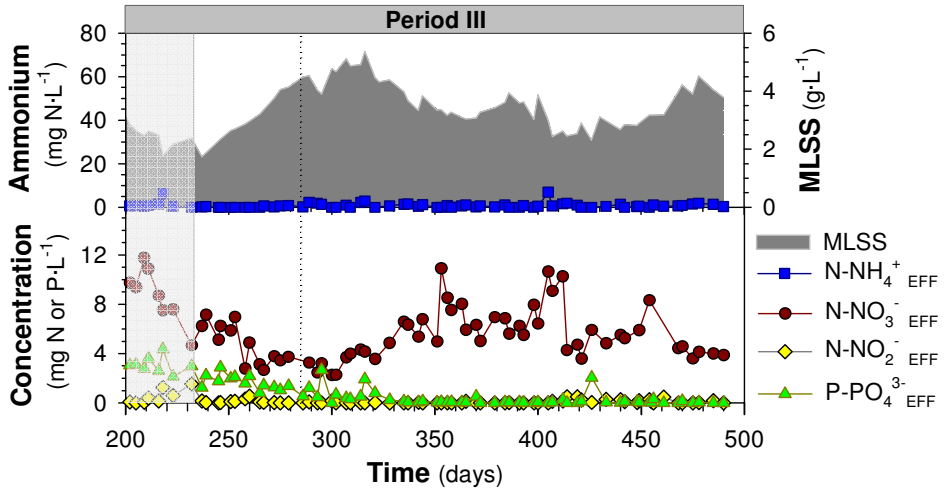

Figure 4.9. Ammonium ($NH_4^+{}_{EFF}$) effluent concentrations and MLSS (top) and nitrate ($NO_3^-{}_{EFF}$), nitrite ($NO_2^-{}_{EFF}$) and phosphate ($PO_4^{3-}{}_{EFF}$) effluent concentrations (bottom) during Period III. Shadowed area corresponds to Period II.

With this cycle configuration, ammonium was completely oxidized (< 1 mg N-$NH_4^+ \cdot L^{-1}$) and nitrate in the effluent decreased from 9.3 to 3.3 mg N-$NO_3^- \cdot L^{-1}$ on day 260. Phosphate also decreased from 3.09 to 1.08 mg P-$PO_4^{3-} \cdot L^{-1}$ (Figure 4.9, bottom) and reached 94% and 74% of nitrogen and phosphorus removal, respectively, at the beginning of Period III. From day 250 on the standard limits requirement of 15 mg $N \cdot L^{-1}$ and 2 mg $P \cdot L^{-1}$ were accomplished.

Nutrient removal was improved after changing the feed pattern from 50%-50% to 70%-30%. This strategy improved nitrogen removal because, as analysed in Table 4.2, nitrate concentration was reduced in the effluent while maintaining ammonium removal. Despite low values of phosphate at the effluent, they were higher than zero, so phosphorus removal efficiency could still have been increased. Cycle analyses were carried out to obtain information about nutrient rates and behaviors. Based on this information, the cycle could be optimized. Figure 4.10 represents the pH profile, nitrogen and phosphorus concentrations and the inorganic and organic carbon obtained from a cycle study on day 279 (Figure 4.9, Period III), right after the recovery of the BNR.

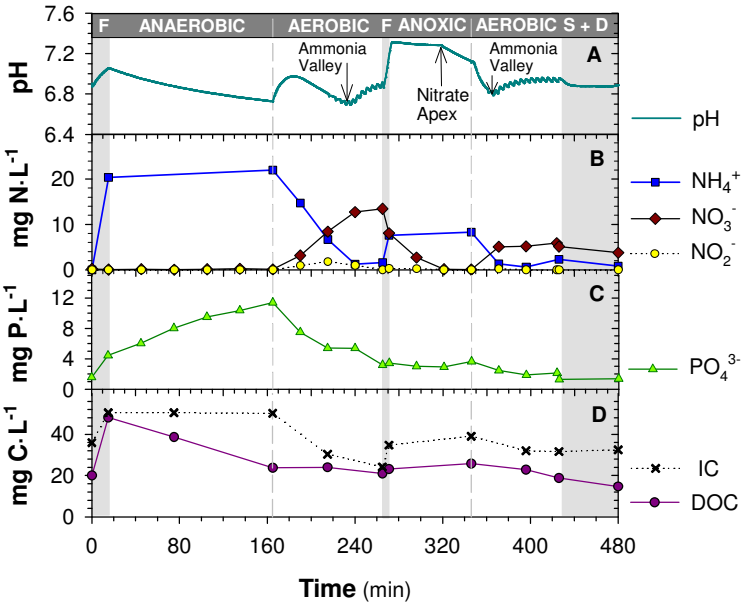

Figure 4.10. Eight-hour cycle profile of pH (A), ammonium (N-NH_4^+), nitrate (N-NO_3^-)and nitrite (N-NO_2^-) (B), phosphate (P-PO_4^{3-}) (C) and inorganic (IC) and dissolved organic carbon (DOC) (D) at day 279 of operation. Shadowed areas correspond to filling and decant phases.

In the first anaerobic phase after the feeding period, the phosphorus profile (Figure 4.10C) did not show phosphate release stabilization, and neither did the pH profile as would happen if phosphate concentration had remained constant. Even though, dissolved organic carbon (DOC, Figure 4.10D) had nearly reached the minimum concentration at the end of the first phase (23.8 mg C·L^{-1}). This remaining DOC can be considered as mostly non-ready biodegradable organic matter as it was not reduced in the subsequent aerobic phase.

With regard to the bioreactions taking place in the aerobic phase, ammonium concentration decreased from 22.0 to 1.2 mg N-NH_4^+·L^{-1}. The end of nitrification was detected by *ammonia valley* (AV) in the pH profile (Figure 4.10A). During ammonium oxidation, nitrate concentration increased to 13.7 mg N-NO_3^-·L^{-1} (Figure 4.10B). The differences observed between the N-NH_4^+ removed and the N-NO_3^- generated in the aerobic phase is explained by a maximum SND efficiency of 35% (see section 2.5 and 3.4.4). A maximum of 1.77 mg N-NO_2^-·L^{-1} was observed in the media under aerobic conditions, which accounted for 0.0025 mg N-HNO_2·L^{-1}. In the same aerobic phase, phosphorus was taken up.

Phosphorus uptake was suddenly stopped at minute 215 because of inhibition when the maximum FNA was reached (Figure 4.8). Phosphate was again taken up after nitrite was totally oxidized to nitrate. Finally, the system achieved a poor PUR (2.0 mg $P\text{-}PO_4^{3-}\cdot g^{-1}$ VSS$\cdot h^{-1}$) compared with the one obtained in Period I without nitrite presence (11.4 mg $P\text{-}PO_4^{3-}\cdot g^{-1}$ VSS$\cdot h^{-1}$), giving an effluent of 1.4 mg $P\text{-}PO_4^{3-}\cdot L^{-1}$. Furthermore, under aerobic conditions, and as a consequence of nitrification, inorganic carbon was reduced from 50 to 24 mg IC$\cdot L^{-1}$ (Figure 4.10D).

The anoxic stage of the cycle later allowed denitrification of this nitrate. The end point of denitrification process was observed by *nitrate apex* (NA), an inflection point in the pH profile (Figure 4.10A), followed by the final aerobic phase where 7.5 mg $N\text{-}NH_4^{+}\cdot L^{-1}$ introduced in the second feed was completely oxidized. In this part of the cycle, 32% of maximum SND was achieved and 5.1 mg $N\text{-}NO_3^{-}\cdot L^{-1}$ remained at the end of the aerobic period. At the end of the cycle, nitrate concentration was reduced to 3.7 mg $N\text{-}NO_3^{-}\cdot L^{-1}$ in the effluent (Figure 4.10C). This was due to denitrification during the settling period, because of remaining organic matter or endogenous denitrification.

It can be seen from both the on-line and the off-line data that aerobic phases were oversized at that stage. Ammonium was oxidized in less than 30 minutes in the second aerobic phase (Figure 4.10B), while the length of the anaerobic phase was not enough to stabilize the phosphate release. In order to optimize the cycle and achieve higher removals, the anaerobic phase was increased by 10% (from 160 to 200 minutes) instead of the second aerobic phase (Table 4.1) at day 285 of operation.

Since anaerobic phase was lengthened, carbon uptake increased and so PHA formation, improving phosphorus removal. The system achieved a stable state from day 290 to the end of the study, with efficiencies of 91%, 92% and 96% for organic matter, nitrogen and phosphorus removal, respectively (Figure 4.3). Ammonium and phosphorus concentrations in the effluent were close to zero, while nitrate achieved the predicted values after the change in feed distribution (Figure 4.9, Table 4.2). Finally, Figure 4.11 shows the pH (A),

nitrogen (B), phosphorus (C) and carbon profiles during an 8h cycle on day 449 at the end of the experiment after a correct long-term operation.

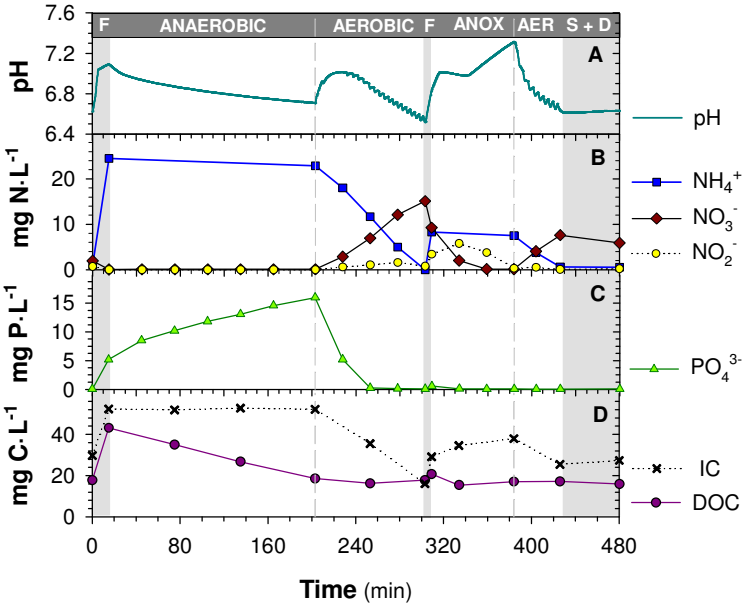

Figure 4.11. Eight-hour cycle profile of pH (A), ammonium (N-NH_4^+), nitrate (N-NO_3^-)and nitrite (N-NO_2^-) (B), phosphate (P-PO_4^{3-}) (C) and inorganic (IC) and dissolved organic carbon (DOC) (D) at day 449 of operation. Shadowed areas correspond to filling and decant phases.

At the beginning of the cycle, phosphate (Figure 4.11C) and DOC (Figure 4.11D) concentrations were not stable at the end of the anaerobic conditions, and neither was the pH profile (Figure 4.11A). However, the final value of DOC (18.6 mg $C{\cdot}L^{-1}$) was close to the remaining concentration present in the aerobic periods. This value was associated with the non-biodegradable organic matter present in the influent wastewater. In the subsequent aerobic period, phosphate was taken up at a PUR of 7.64 mg $P\text{-}PO_4^{3-}{\cdot}g^{-1}$ $VSS{\cdot}h^{-1}$, a higher value than the rates obtained at the beginning of Period III (2.0 mg $P\text{-}PO_4^{3-}{\cdot}g^{-1}$ $VSS{\cdot}h^{-1}$). The maximum nitrite concentration reached under aerobic conditions was 1.59 mg $N\text{-}NO_2^-{\cdot}L^{-1}$, despite the FNA only reaching 0.0005 mg $N\text{-}HNO_2{\cdot}L^{-1}$ during phosphate uptake. The increase in PUR could have been due to either an increase in PHA during the anaerobic phase, or an enrichment of the PAO population because FNA inhibition was avoided. The DOC taken up had similar

values in both cycles (24.6 and 24.1 in Figures 4.10D and 4.11D, respectively). As is shown in the next section, the increase in the PAO population was responsible for the enhancement of EBPR performance.

With regard to nitrogen compounds (Figure 4.11B), ammonium took the entire first aerobic phase to be reduced below the detection level (< 0.1 mg $N\text{-}NH_4^+ \cdot L^{-1}$), while nitrate increased to 15.8 mg $N\text{-}NO_3^- \cdot L^{-1}$. Nitrogen differences between the ammonium removed and nitrate generated gave a maximum SND efficiency of about 31%. In the same aerobic phase, IC was reduced from 52.1 to 16.1 mg $C \cdot L^{-1}$ (Figure 4.11D). Nitrate was completely removed by heterotrophic denitrification under the following anoxic conditions. In the second aerobic phase, 7 mg $N\text{-}NH_4^+ \cdot L^{-1}$ were removed, with nitrate increasing to 7.3 at mg $N\text{-}NO_3^- \cdot L^{-1}$, so only 5% of maximum SND was observed in the absence of phosphate. The differences in SND activity were not attributable to heterotrophic denitrification, as variations in organic matter (DOC, Figure 4.11D) in both aerobic phases were under the detection limit (< 1 mg $DOC \cdot L^{-1}$). This means SND was related to EBPR improvement, as SND was mainly observed when phosphorus uptake was taking place. This was probably due to simultaneous denitrification and phosphorus removal being carried out by denitrifying PAOs or DPAOs. Finally, all the analyses show that all the processes were working efficiently for biological nutrient removal, with about 88%, 93% and 99% of carbon, nitrogen and phosphorus removal, respectively, being obtained.

4.4.4 Evolution of microbial population

Microbiological analyses were periodically performed using the FISH technique to determine the presence of nitrogen and phosphorus removal microbial population. PAO and GAO were analyzed to evaluate the EBPR process. AOB and NOB were checked to follow the enrichment of nitrogen removal bacteria. Table 4.3 summarizes the percentages obtained in terms of the total bacteria (see section 3.3.4.2) during the study.

Table 4.3. Microorganisms' population during the study. Percentages are calculated over total bacteria analyzed by FISH. NA: Not Analyzed.

Period	Day of Operation	PAO	GAO	AOB + NOB
		%	%	%
I	0	15 ± 2	3.5 ± 1	NA
II	156	16 ± 3	23 ± 3	2 ± 1
	190	17 ± 2	35 ± 2	4 ± 1
III	427	63 ± 4	1 ± 1	9 ± 2

On day 156, when nitrification had just been restored, nitrifiers, as the sum of AOB and NOB, accounted for 2% of the total bacteria population, PAOs accounted for 16% and GAOs 23%. On day 190, once nitrification had recovered, the nitrifiers increased to 4%, PAOs remained stable at 17% and a significant increase in GAOs from 23% to 35% was observed (Table 4.3). Some of the FISH micrographs obtained during the microbiological analyses for phosphorus and nitrogen removal are depicted in Figure 4.12.

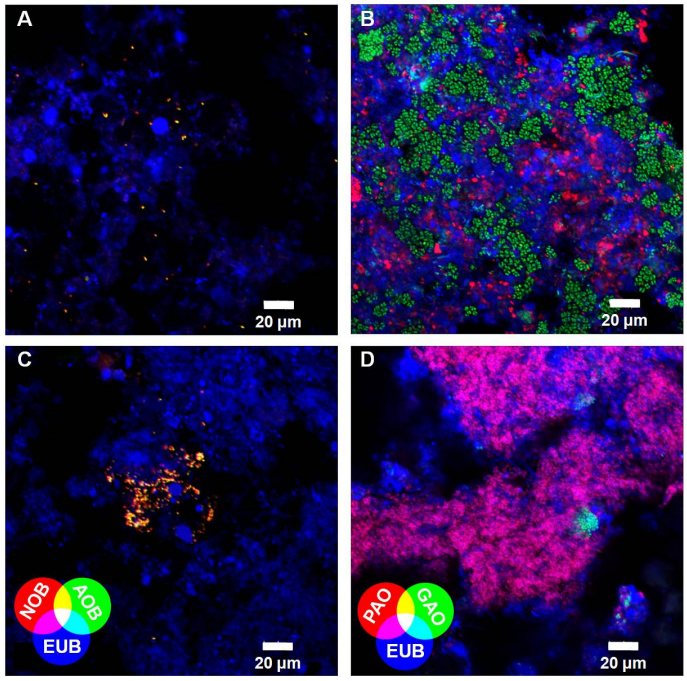

Figure 4.12. FISH micrographs for AOB (green) and NOB (red) on day 190 (A) and 427 (C); PAO (red) and GAO (green) on day 190 (B) and 427 (D). All bacteria (EUB) were stained in blue.

Organic matter competition must be avoided to provide PAOs microorganism with enough energy. Because of that, no nitrate should be present in anaerobic conditions, as denitrifiers are the most common competitors for organic matter with phosphorus removal population. This was the case of Period II where nitrate was not removed from the system and remained under anaerobic conditions. Furthermore, according to Filipe *et al*. (2001), GAOs are able to anaerobically take up volatile fatty acids (VFA) faster than PAOs below a pH of 7.25. According to cycle profiles (Figure 4.10A and 4.11A) the maximum pH achieved under anaerobic phase was 7.2. Finally, the effect of FNA on the aerobic performance of PAO microorganisms (Figure 4.8) reduced their activity and, as a consequence, their growth. Saito *et al.* (2004) have suggested that nitrite accumulation provides GAOs with an advantage over PAOs. For these reasons, PAOs population remained stable while GAOs were increased in Period II.

In Period III the cycle was modified in order to enhance phosphorus removal. This change was also the turning point for the EBPR microbial population, as the system started to become enriched with PAOs (63%) to the detriment of GAOs (1%). A FISH analysis of nitrifiers on day 427 (Table 4.3) also revealed an enrichment of autotrophic bacteria to 9%, double of the previous concentration obtained on day 156.

After more than four months, the system was stable and achieving concentrations below the discharge levels (41 mg $COD \cdot L^{-1}$; 3.4 mg $N \cdot L^{-1}$; 0.27 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$). FISH analysis confirmed operational conditions were suitable for achieving higher efficiencies leading to an enrichment of the bacteria responsible for nutrient removal.

4.5 Discussion

4.5.1 Aerobic distribution affecting SRT

A change in the reactor operation affecting the influent characteristics (i.e. an increase of load) can lead to a complete destabilization of the nutrient removal

performance. The SBR flexibility allows adjusting the operational conditions in order to reestablish the reactor operation. Nitrogen and phosphorus removal require different operational conditions to be carried out. Therefore, when disturbances are observed in BNR, different steps should be applied to restore the system. Nitrification is the most sensitive process after a loading increase, as also stated Hu *et al.* (2010), because of the slow growth of nitrifying bacteria. It is the first process to be recovered as it is severely affected by a low SRT, which can happen when insufficient biomass has grown to compensate for the loading increase. When ammonium oxidation goes up, nitrate in the reactor increase. Denitrification efficiency is the following target, as nitrate is the major compound interacting with phosphorus removal. Puig (2008) stated that after a system stabilization using floccular sludge the system returns to normal conditions, first, recovering the denitrification efficiencies and after 15 days the phosphorus removal. Accordingly, EBPR should be also the last goal of the stabilization strategy when working with granular sludge.

When trying to improve nitrification, an SRT_{AER} over four days is essential. This can be achieved by increasing the aerobic phase at the expense of part of the anaerobic phase. However, transient response periods when conditions are being changed (from anoxic to aerobic) have to be considered as they can limit the growth of the nitrifiers. Reducing the step-feed events by joining together the aerobic phases, thereby obtaining a longer aerobic period, will achieve maximum bacterial activity and improve ammonium removal from the media. Furthermore, diffusion phenomena around the activated sludge flocs (and even more so in the presence of granular sludge) might be a determining factor in the transient phenomenon. Even with a similar SRT, longer aerobic periods and reduced alternating conditions enhance ammonium oxidation after a load increase as transient response periods are avoided. Moreover, this strategy has been proved to favor nitrifying bacteria growth according to FISH analysis.

4.5.2 Feed volume distribution for nitrogen and phosphorus removal

After nitrification is recovered in an SBR, the presence of nitrate and/or nitrite in the anaerobic phase destabilizes the EBPR performance due to organic matter competition. Furthermore, when nitrite is the intermediate of nitrification-denitrification in the system, inhibition by FNA would affect

phosphorus removal severely reducing the PUR at short-term and the PAO population at long-term. Hence, the wastewater distribution of the feeding events increasing the first feed to the detriment of the second is one of the possible solutions tested in this chapter. A higher volume distribution in the first part of the cycle makes organic matter available for EBPR while less nitrate concentration is obtained in the effluent. With regards to phosphorus removal, anaerobic length has to be adjusted after increasing the volume fed in order to allow all the organic matter to be taken up and phosphate release stabilization.

4.5.3 Simultaneous processes

The spontaneous formation of some granules in the reactor can occur when loadings are increased and biomass is washed out (the natural selection of bigger particles). Granules allow different conditions (anaerobic, anoxic and aerobic) in the same tank due to oxygen diffusion limitation. Because of this, simultaneous processes such as SND or even simultaneous nitrification, denitrification, and phosphorus removal (SNDPR) can be obtained and more easily controlled than in activated sludge flocs (Mosquera-Corral *et al*., 2005). A combination of denitrification and EBPR in one process could offer substantial savings on carbon for the overall nutrient removal process, which makes this approach highly attractive (Meyer *et al*., 2005). Non-controlled granular formation improved the performance of the BNR in this study by SNDPR. Therefore, the new step to enhance domestic wastewater treatment and avoid organic matter limitations could be the application of the granular sludge technology for nutrient removal.

4.6 Conclusions

The exposure of SBR systems to permanent high loading rates, for example because of an increase of population or during summer periods, can seriously destabilize BNR performance. The disruption in nitrogen and phosphorus removal cannot be solved by applying the same operational conditions; BNR recovery must follow the step-wise procedure. The practical experience gained during this study shows that longer aerobic phases enhance the recovery of

nitrification, in spite of working in split aerobic phases at the same aerobic SRT. Once ammonium oxidation has been established, special attention must be paid to the wastewater distribution of a step-feed SBR. An increase in the first feed volume would reduce nitrate accumulation and prevent substrate competition, which would enhance *Competibacter* growth.

Optimal configuration can only be worked out when nitrogen and phosphorus removal have been achieved. Adjusting the length of some of the phases can help to raise efficiency rates and bacterial population growth. As a general conclusion, there is no such thing as a perfect SBR operation; changes have to be gradually applied depending on the state of the BNR system. This means that a dynamic operation is required for BNR processes and this is easily obtained with SBR technology.

Chapter 5. Granular sludge with low-strength wastewater: development strategies and nutrient removal application

Granules can improve the nutrient removal performance as it has been observed from previous research in this thesis. This chapter is focused on the challenge of granular sludge formation for nutrient removal treating low-strength wastewater. Different strategies were applied to develop granules based on settling time decrease and different volume exchange ratios in order to increase the organic loading rate. When a fully granular reactor was obtained, biological nutrient removal efficiency was evaluated. Finally, in order to assess the interactions of nitrogen and phosphorus removal, different tests were carried out for simultaneous processes within the granules. Nitrate and nitrite as products of nitrification were investigated for simultaneous denitrification and phosphorus removal.

This chapter is based on the following publication:

Coma, M., Puig, S., Balaguer, M.D., Colprim, J. 2010. The role of nitrate and nitrite in a granular sludge process treating low-strength wastewater. *Chem. Eng. J.* **164** (1): 208-213.

Coma, M., Puig, S., Serón, N., Balaguer, M.D., Colprim, J. 2009. Granular sludge development at different exchange ratios for nutrient removal. *2nd IWA Specialized Conference on Nutrient Management in Wastewater Treatment Processes.* (6-9 September, Krakow, Poland).

5.1 Introduction

Sequencing batch reactors (SBRs) have been extensively studied in the context of biological nutrient removal (BNR). To improve the performance of this technology, the biomass can be compacted by sludge granulation. Granules are dense sludge aggregates with good settleablility and a high biomass concentration. The cultivation of granules in SBR systems offers a new tool with which to improve wastewater treatment. The characteristics of the granules make compact systems possible, thereby reducing the need for the long settling times and large surface areas associated with conventional activated sludge systems. The development of the granules is one of the key points in the process and various parameters have been identified as factors affecting their formation (Liu and Tay, 2004, see section 2.7).

Granular sludge has been studied since it was first reported in an SBR by Morgenroth *et al.* (1997). However, spontaneous granulation of suspended biomass has mainly been observed in SBRs applying short fill periods (McSwain *et al*., 2004b) and most of these studies were carried out with high- or middle-strength synthetic wastewater (Ni *et al*., 2009). Organic loading rates (OLRs) between 2-20 Kg COD·$m^{-3}d^{-1}$ have been applied for granular development (Adav *et al*., 2010b). However, when working with low-strength wastewater such as the domestic type, a low OLR (less than 1 Kg COD·$m^{-3}d^{-1}$) results in slower formation and a longer time to reach a steady state (Tay et al., 2004).

BNR is widely applied in wastewater treatment (Tchobanoglous *et al*., 2003). However, it requires different conditions to be carried out, sometimes in a single reactor, resulting in the coexistence of diverse population and dealing with substrate competition. Granular sludge has been extensively studied for organic matter removal, but little work has been reported for BNR where different bacteria must coexist in the same granule (Etterer and Wilderer, 2001; Cassidy and Belia, 2005; Lemaire *et al.*, 2008b). Most of the studies carried out have involved high organic loading rates, but none of them obtained granules for BNR treating low-strength wastewater.

5.2 Objectives

A fully granular system is difficult to acquire with low organic loading rates, but spontaneous sludge aggregation can be obtained when reactors are stressed, as was observed in Chapter 4. This chapter aims principally to evaluate the feasibility of obtaining granules from a low-strength stream by applying different strategies involving a settling time reduction and/or a variation in the volume exchange ratio. The applicability of a granular reactor for nutrient removal and the interaction within the reactions is also assessed.

5.3 Experimental procedure

5.3.1 Lab-scale set-ups

Three 30 L lab-scale SBRs (see section 3.1.1) were used in the experiments. They were seeded with activated sludge from the Sils-Vidreres wastewater treatment plant (WWTP) (Girona, Spain) and fed with synthetic wastewater (see section 3.2) based on organic matter (498-615 mg $COD \cdot L^{-1}$), ammonium chloride (69 – 85 mg $N \cdot L^{-1}$) and phosphate buffer (5.9 – 6.4 mg $P \cdot L^{-1}$). The influent was always introduced under anaerobic or anoxic conditions to improve phosphorus removal and denitrification. A sequence of feed-anaerobic-aerobic phases followed by a second sequence of feed-anoxic-aerobic phases was used, focusing on organic matter, nitrogen and phosphorus removal. The initial settling time was set at around 40 minutes and reduced to 2 minutes during the study. The time extracted from the settling period was added to the aerobic phases. The cycle ended with a 15-minute decant phase. The on/off dissolved oxygen (DO) set-point was fixed at 1.5 mg $O_2 \cdot L^{-1}$ for all SBRs. Figure 5.1 shows the scheme of the eight-hour cycle applied in the SBRs.

Figure 5.1. Eight-hour cycle applied in the SBRs for nutrient removal purposes.

The volume exchange ratio (VER) was modified from SBR-1 to SBR-2 by decreasing the minimum volume and increasing the influent introduced. From SBR-2 to SBR-3, the VER was modified increasing the volume of wastewater introduced in the reactor while maintaining the high of decant. VERs were set at 40% for SBR-1, 50% for SBR-2, and 60% for SBR-3. This increased the minimum settling velocity of the sludge in order to be kept inside the reactor, obtaining 4.9 $m \cdot h^{-1}$, 5.4 $m \cdot h^{-1}$ and 8 $m \cdot h^{-1}$ for SBR-1, SBR-2 and SBR-3, respectively after 2 minutes of settling time. All this parameters and the hydraulic retention time (HRT) and the organic carbon (OLR), nitrogen (NLR) and phosphorus (PLR) loading rates applied in each reactor are summarized in Table 5.1.

Table 5.1. Operational conditions applied in each reactor during the experimental study.

	Vmin	VER	HRT	OLR	NLR	PLR
	L	%	Hours	Kg COD·$m^{-3}d^{-1}$	g N·$m^{-3}d^{-1}$	g P·$m^{-3}d^{-1}$
SBR-1	16	40	20	0.6 ± 0.1	93 ± 27	7 ± 2
SBR-2	12	50	16	0.8 ± 0.1	104 ± 15	10 ± 3
SBR-3	12	60	10	1.0 ± 0.2	175 ± 27	13 ± 6

5.3.2 Batch experiments set-up

Four batch tests were carried out in 5 L sealed glass reactors (see section 3.1.2) using 2 L of granular sludge taken from the parent SBR (SBR-1) at the end of the aerobic phase. The reactors were fed with synthetic wastewater composed of acetate (as organic matter source), ammonium (to avoid assimilation

limitations due to heterotrophic growth), phosphate and microelements. The sludge was exposed to anaerobic conditions for 190 minutes followed by 150 minutes of anoxic or aerobic conditions. After the anaerobic phase, a pulse of nitrate (A) or nitrite (B) of around 20 mg $N \cdot L^{-1}$ was fed in the anoxic phases. This concentration was chosen because it was the maximum amount of nitrogen achieved inside the granular SBR during the whole study. Oxygen was supplied with different concentrations in both aerobic conditions for test C and D (1.5 and 7.0 mg $O_2 \cdot L^{-1}$, respectively). Table 5.2 summarizes the initial conditions for each experiment.

Table 5.2. Initial conditions for batch test studies.

Test	Conditions	Carbon source $[mg\ C \cdot L^{-1}]_0$	Ammonium $[mg \cdot L^{-1}]_0$	Phosphate $[mg\ P \cdot L^{-1}]_0$	Electron acceptor compound	$[mg \cdot L^{-1}]_0$
A	anoxic	43	4.9	3.4	Nitrate	20.7
B	anoxic	40	4.9	3.5	Nitrite	25.2
C	aerobic	38	4.9	3.6	Oxygen	1.5
D	aerobic	41	4.9	3.5	Oxygen	7

The systems were thermostated at 20.0 ± 0.5ºC. The pH range varied from 7.3 to 8.2. The ratio between the anoxic phosphate uptake rate (PUR) and the aerobic PUR were used as an index of denitrifying phosphorus accumulating organisms (DPAOs) activity (Tsuneda *et al.*, 2006).

5.4 Results

This chapter is based on the assessment of granular sludge for nutrient removal when treating low-strength wastewater. First, different strategies for developing granules were evaluated. Then, when the most suitable conditions for granulation had been applied, BNR and the interactions between bioprocesses within the aggregates were investigated.

5.4.1 Strategies for aerobic granular sludge development

Aerobic granules have usually been cultivated in medium- and high-strength streams with high OLRs. When working with low-strength wastewater in SBRs, the OLR can only be increased by raising the volume exchange ratio (VER). For this reason, three SBRs were started up for granulation purposes and different VERs were applied (40%, SBR-1; 50%, SBR-2 and 60%, SBR-3). The reactors were run for 20 days after inoculation for acclimation purposes and the granulation strategy was started at day 0 of operation. Figure 5.2 shows the OLRs applied in the SBRs during the entire whole time of the experiment.

The OLR applied in these reactors was between 0.5 and 1 Kg COD·$m^{-3}d^{-1}$. These values are far below the minimum of 2 and maximum of 20 Kg COD·$m^{-3}d^{-1}$ cited in the literature for granulation purposes (Adav *et al.*, 2010b), even working at 60% VER. The variation in OLR during the study (Figure 5.2) was due to organic matter degradation in the storage tank, even though the synthetic wastewater was prepared twice a week and maintained at 4ºC.

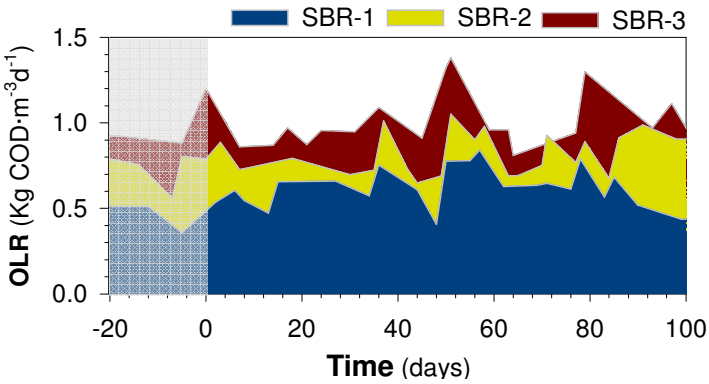

Figure 5.2. OLR applied in SBR-1 (40% VER), SBR-2 (50% VER) and SBR-3 (60% VER) during the experimental study. Shadowed area corresponds to acclimation period.

5.4.1.1 Settling time reduction

After 20 days of acclimation (from -20 to 0 days, Figure 5.2) the granulation strategy was started on day 0 of the study. When selecting granules from floccular sludge, short settling times have to be applied. To avoid large biomass

washout, a sequential methodology for a settling time decrease was analyzed using exponential expressions such as ts = 1 + a·e^{-kt}, with "a" being the initial settling time and "k" a constant which changes the settling decrease profile. Figure 5.3 illustrates the theoretical evaluation and the experimental settling time decrease applied in the SBRs.

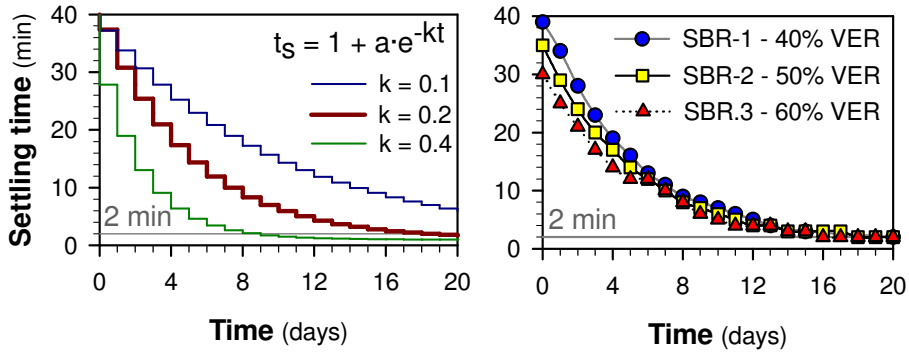

Figure 5.3. Theoretical (left) and experimental (right) decrease in settling time applied in the three SBRs.

Different constants were evaluated to decrease the settling time to 2 minutes in around 20 days. A constant of k = 0.2 was chosen to represent the smoothest profile while achieving the desired settling time within the aimed period (Figure 5.3, left). The initial settling times of the reactors were 40, 35 and 30 minutes for SBR-1, SBR-2 and SBR-3, respectively. Initial settling time was fixed according to settling velocities of each biomass and maintained during the acclimation period. The settling time was decreased exponentially in line with the profile defined by the mathematical expression chosen, following which settling time of 2 minutes was achieved in all three SBRs after 20 days. This methodology was adopted to reduce biomass washout and faster particles were selected, but still floccular biomass was removed from the reactor, with the result that the loss of mixed liquor suspended solids (MLSSs) was unavoidable. Figure 5.4 shows the biomass concentration profiles set against the settling times during the whole of the study.

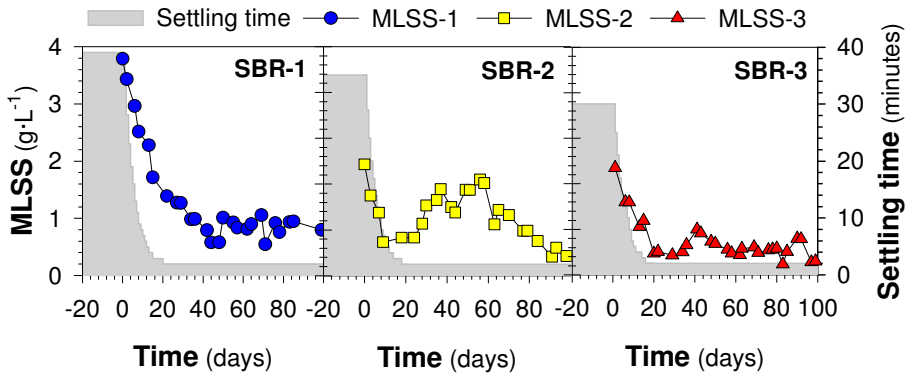

Figure 5.4. MLSSs and settling times in SBR-1 (40% VER), SBR-2 (50% VER) and SBR-3 (60% VER) during the experimental period.

The biomass concentrations at the beginning of the experimental study (day 0) were 3.8, 2.4 and 2.4 g MLSS·L^{-1} for SBR-1, SBR-2 and SBR-3, respectively. From day 0 to day 20, the settling times and solids concentrations in all reactors were reduced. Immediately after the settling was set at 2 minutes, SBR-1, SBR-2 and SBR-3 presented 1.7, 0.8 and 0.5 g MLSS·L^{-1}, respectively. Different behaviors were seen in terms of biomass concentration during the following operation. In the case of SBR-1, applying a VER of 40%, a smooth reduction of MLSS from 1.7 to 0.8 g·L^{-1} was achieved due to the progressive washout of slow settling particles. In SBR-2, applying a VER of 50% meant a higher OLR was obtained, thus making more organic matter available for heterotrophic microorganism growth. As a result, solids recovered to 2.0 g MLSS·L^{-1} until day 60 when filamentous bacteria started to appear in the system and caused a biomass washout with 0.4 g MLSS·L^{-1} being achieved at the end of the operational period. Finally, SBR-3, which was working at the highest OLR (1 Kg COD·$m^{-3}d^{-1}$), presented no biomass growth. Instead, solids were continuously reduced by effluent discharge and 0.3 g MLSS·L^{-1} were obtained at the end of the study. This could have been due to the bigger VER applied, which apart from providing greater OLRs, would have created pressure during the draw period due to the higher volume to be discharged.

5.4.1.2 Physical and morphological study of granular sludge

Biomass was washed out from the reactors due to the settling time reduction, but granules could be seen by the naked eye immediately after settling was fixed at 2 minutes in all reactors. The particles formed were analyzed in terms of size and morphology. Figure 5.5 shows the size distribution in terms of particle percentages from all the SBRs, while Table 5.3 summarizes the morphological characteristics (roundness or aspect ratio, see section 3.3.3.2) of the granules obtained.

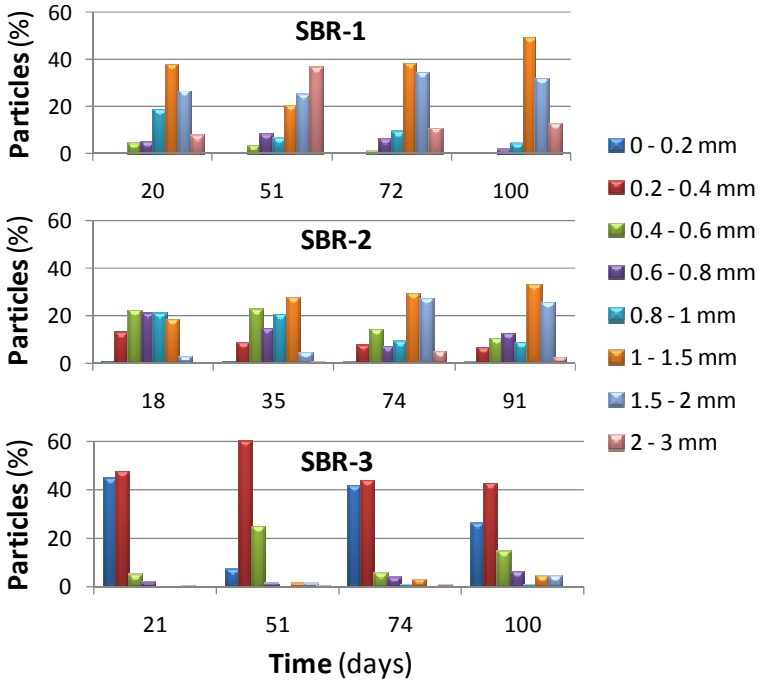

Figure 5.5. Size distribution in particle percentages for SBR-1, SBR-2 and SBR-3 during the study.

The biggest granules, as can be seen in Figure 5.5, were obtained from SBR-1 working at 40% VER. Particles over 2 mm were found around day 51, but these broke down and granules remained mainly between 1 and 2 mm during the rest of the study. These particles had an amorphous aspect; they were not completely spherical (a theoretical roundness factor of 1) as shown by their roundness value of 0.7 for the whole study (Table 5.3). This is in agreement

with de Kreuk and van Loosdrecht (2006), who suggested that irregularity would be more common when fewer granules were present, resulting in fewer collisions and a reduction in the shear stress on the granules.

Table 5.3. Morphological characteristics of the granules.

	Days	SBR-1	SBR-2	SBR-3
Roundness	20-30	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
	50-60	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
	80-90	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.2

In SBR-2, where 50% VER was applied, a progressive growth in particle size was observed from the day settling time was fixed to the end of the process. At day 18 (Figure 5.5) particles from 0.2 to 1.5 mm were observed, but these increased substantially over 1mm at day 91. The granules had roundness values of 0.7 (Table 5.3), indicating an incomplete spherical aspect. SBR-3, which was fed with the highest volume of wastewater (60% VER) had the smallest particles in the study, with a size distribution mainly around 0.2 mm (Figure 5.5). These small particles were the ones that had a more spherical morphology immediately after the decrease in settling time, with a roundness value of 0.8. However, this value decreased to 0.7 through the study, although higher deviations indicating a more heterogeneous sample were also obtained (Table 5.3).

Settling time reduction caused granules to appear in all three systems. Nevertheless, when the shortest settling time was reached on day 20, floccular biomass was still being removed (Figure 5.4) and hence the settling properties of the remaining sludge improved. To evaluate the settleability of the sludge and enable comparison with granule formation, Figure 5.6 shows the sludge volumetric index (SVI) and the mean size of the particles obtained. The SVI from SBR-2 was not analyzed.

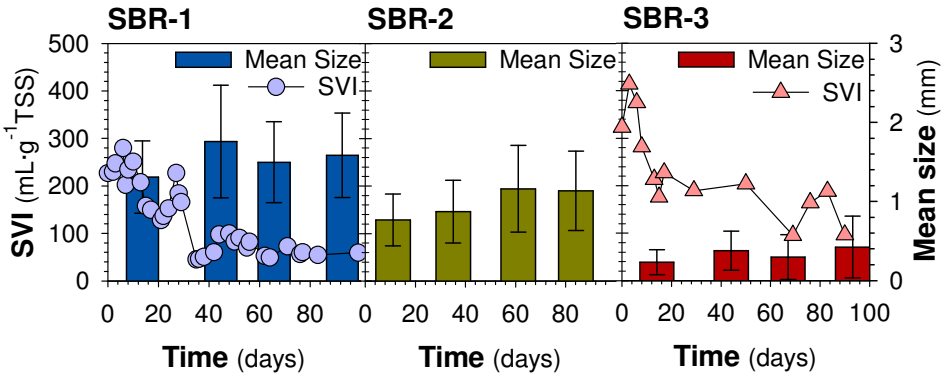

Figure 5.6. Sludge volumetric index (SVI) and mean size of granular particles during the study.

The SVI initially observed presented values of over 200 mL·g^{-1} TSS in SBR-1, which corresponds to floccular sludge. These values started to decline when settling time was reduced (from day 0 to 20, Figure 5.6). In the case of SBR-1, SVI was stabilized at 67 ± 18 mL·g^{-1} when the mean size of the granules were around 1.5 mm. This coincided with the complete removal of floccular sludge and the system becoming a fully granular reactor. The granules present in SBR-2 showed mean size values of over 1 mm after day 60. Finally, SBR-3 presented even higher SVI values of 320 mL·g^{-1} due to filamentous bulking. When applying a higher VER (60%), the bigger volume of discharge would reduce the biomass concentration from the reactor. As a consequence, organic matter would be more available for filamentous bacteria growth. An overgrowth of filamentous bacteria can entrap the granules from the reactor damaging the good settling properties of the system, even more if they present small sizes such as SBR-3 (0.5 mm).

To obtain a physical evaluation of all three reactors, stereomicroscope images were taken regularly. Figure 5.7 shows some of the images from the mixed liquor of each reactor during the study.

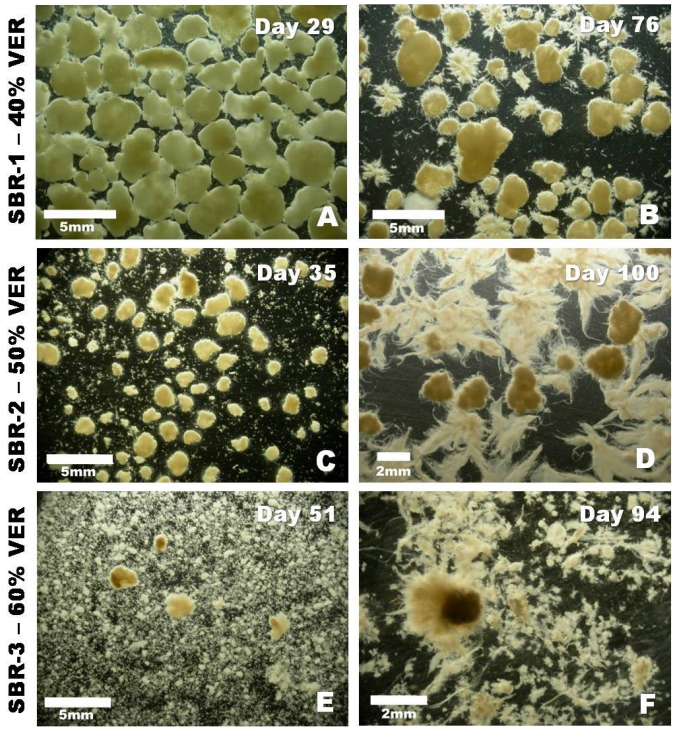

Figure 5.7. Stereomicroscope images from the three SBRs during the experimental study. A and B: SBR-1; C and D: SBR-2; E and F: SBR-3. Scale bars: 5 mm for A, B, C, and E; 2 mm for D and F.

It can be seen from the images that SBR-1 was the only reactor which was fully granular throughout the whole study (Figure 5.7, A and B). However, from the images of the last days of the reactor's operation (Figure 5.7B), it can be observed that there were some filamentous bacteria around the granules, although they did not affect the reactor's settling properties (SVI of 59 ± 7 $mL \cdot g^{-1}$TSS). Regarding SBR-2, some floccular sludge persisted in the system during the first days of the study, even though huge size differences between flocs and granules were observed (Figure 5.7C). In contrast, an outgrowth of filamentous bacteria did not allow granules to remain inside the reactors after day 100 (Figure 5.7D), as they remained entrapped between the filaments. In the case of SBR-3, sludge particles were a minimal part of the biomass. Figure 5.7E shows that some cores were starting to grow among the floccular sludge. Figure 5.7F shows that only a few particles surrounded by filamentous bacteria remained in the reactor, while the majority of the sludge was still floccular.

To sum up, the application of higher VERs of 50% and 60% allowed the OLR to increase in the reactors. At the same time, higher pressure because of water discharge caused a more significant biomass washout than with 40% VER. Because of the minimal solids concentrations within the SBRs, more organic matter was available for filamentous growth, thereby affecting the settleability and stability of granules in SBR-2 and SBR-3. In this study treating synthetic wastewater with the lowest VER of 40% resulted in a granular reactor that was stable in terms of physical parameters.

5.4.2 Biological nutrient removal using aerobic granular sludge

Aerobic granular sludge was achieved when applying VERs from 40% to 60%, but the stability of the particles seemed to be affected by high organic loading rates (high VER). The larger the volume that was treated, the less stable became the system. SBR-1, with a treatment of 40% VER, was the most stable reactor in terms of the physical parameters affecting granular sludge, so its operation was continued in order to evaluate its nutrient removal efficiency.

5.4.2.1 Granular nutrient removal performance

Once granular sludge had been obtained in SBR-1, the reactor was operated at the same conditions for 300 days. Table 5.4 summarizes the mean values applied during the nutrient removal evaluation.

Table 5.4. Operational conditions applied in SBR-1.

	Influent	Loads	HRT	COD_T/N/P
	$mg \cdot L^{-1}$	Kg $COD \cdot m^{-3} d^{-1}$ g N or $P \cdot m^{-3} d^{-1}$	days	g COD:g N:g P
Organic matter (COD)	617 ± 158	0.7 ± 0.2		
Nitrogen (N)	78 ± 8	94 ± 10	0.82 ± 0.02	100:13:1.8
Phosphorus (P)	6 ± 1	8 ± 2		

BNR performance with 40% VER was evaluated. Aerobic granular sludge was able to treat low-strength wastewater removing 86 ± 6 % of organic matter. The loss of biomass from the granular strategy applied (see section 5.4.1.1) had

a strong influence on the sludge residence time (SRT), which affected the nutrient removal. Figure 5.8 shows the efficiencies of total nitrogen and phosphorus removal, as well as MLSS and SRT during the study.

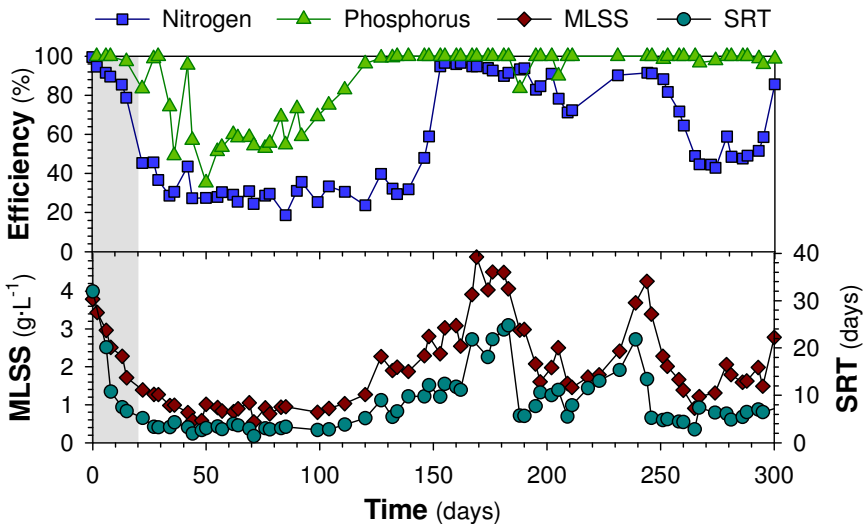

Figure 5.8. Nitrogen and phosphorus efficiencies (top) and MLSS and SRT (bottom) during the study of nutrient removal in granular sludge. Shadowed area stands for settling time reduction period. Shadowed area corresponds to settling time decrease period.

As can be seen in Figure 5.8, nitrogen and phosphorus removal were reduced from nearly 100% to 28% and 35%, respectively, around day 50. This decrease in both efficiencies was caused by the huge washout of biomass from 3.8 g to less than 1.0 g MLSS·L^{-1}. The loss of sludge directly affected the SRT, which had the same profile as that of MLSSs throughout the study (Figure 5.8, bottom). Durging the time when nitrogen and phosphorus presented minimum efficiencies (from day 40 to 110), SRT was decreased to a mean value of 3 days, which was not sufficient for neither nitrogen nor phosphorus removal. The aerobic SRT required for nitrogen removal SRT_{AER}; see section 3.4.2.1) is usually 5.7 days at room temperature (20ºC). A total SRT of between 2 and 5 days is required for phosphorus removal without a nitrification process (Tchobanoglous *et al.*, 2003).

Phosphorus was progressively recovered to nearly 100% while MLSS increased smoothly from 0.8 to 1.9 g MLSS·L^{-1} and SRT rose to 7 days around day 130.

However, nitrogen did not improve until 2.3 g MLSS·L^{-1} and 10 days of SRT (5 days of SRT_{AER}) were achieved around day 150. Phosphorus removal was stable and achieved efficiencies of 99 ± 3 % during the rest of the study, but some disturbances were seen in nitrogen removal at days 210 and 270 due to a reduction in the SRT. In order to evaluate the physical parameters which affected MLSSs concentration from the granular sludge reactor, SVI and the mean size of the granules were calculated and are depicted in Figure 5.9.

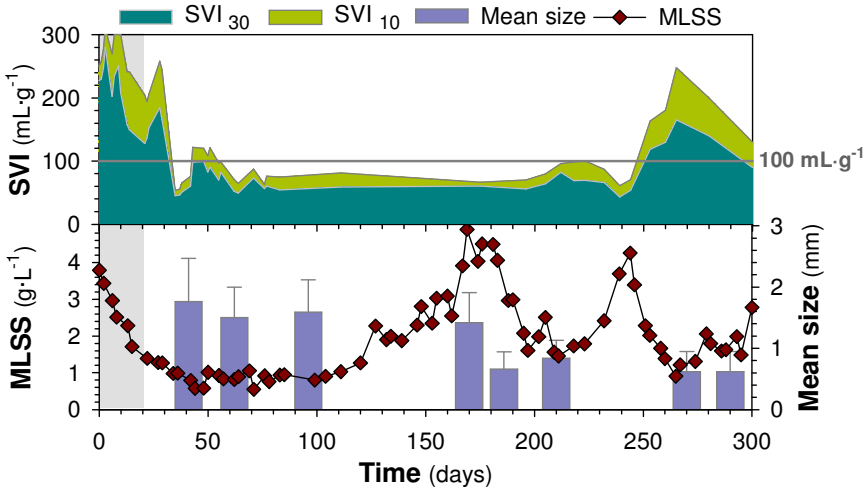

Figure 5.9. Sludge volumetric index at 10 minutes (SVI_{10}) and 30 minutes (SVI_{30}) (top). Mean size of granular sludge and MLSS (bottom) during the study. Shadowed area stands for settling time reduction period.

SVI after 10 minutes of settling (SVI_{10}) in combination with SVI_{30} has been proposed as a way to characterize the settleability of granular activated sludge (Schwarzenbeck *et al.*, 2004). Values close to each other for these parameters indicate granule formation and thickening after settling. Furthermore, Beun *et al.* (2002a) stated that SVI over 100 mL·g^{-1} seems to point to flocculated sludge. Therefore, granules can be considered to have been present for a long time in SBR-1, as similar values of SVI_{10} and SVI_{30} lower than 100 mL·g^{-1} (mean SVI_{30} of 61 mL·g^{-1}) were obtained between day 60 and 250 (Figure 5.9, top).

In terms of the overall MLSS profile (Figure 5.9, bottom), cyclical behavior can be seen during the operational period. Two MLSS peaks were observed, with a solids concentration of 5.0 g MLSS·L^{-1} at day 169 and 4.3 g MLSS·L^{-1} at day 224.

Before the first peak, the mean size of the granules was around 2 mm. Granules were broken and small particles were washed out through the effluent (up to 400 mg TSS·L^{-1}), and the particle size was reduced to 0.8 ± 0.3 mm. The mean size of the granules after the second biomass peak of biomass was 0.6 ± 0.3 mm. During this last part of the study (from day 250 to the end), the difference between SVI_{10} and SVI_{30} became wider and reached values of over 100 mL·g^{-1} (Figure 5.9, top), which indicates poor granule settleability due to their small size. Because their dynamic nature the granules did not present a steady state, which caused MLSS to decrease and, as a consequence, nutrient removal disturbances.

In order to investigate nutrient removal performance and the interactions between nitrogen and phosphorus removal in depth, Figure 5.10 focuses on effluent concentrations of nitrogen (ammonium, nitrate and nitrite) and phosphorus compounds.

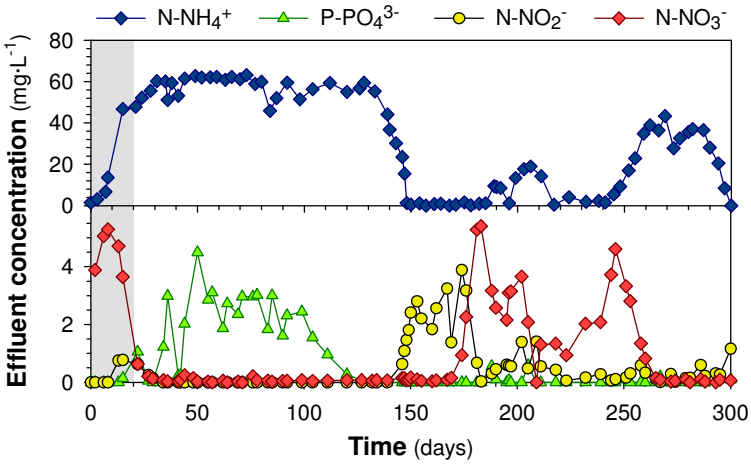

Figure 5.10. Ammonium (NH_4^+) (top) and phosphate (PO_4^{3-}), nitrate (NO_3^-) and nitrite (NO_2^-) (bottom) effluent concentrations during the study of nutrient removal in granular sludge. Shadowed area stands for settling time reduction period.

At day 0 of the study granulation strategies were applied in the system (see section 5.4.1), causing a destabilization in the nitrogen and phosphorus removal processes. Ammonium concentration in the effluent reached a maximum of about 60 mg $N\text{-}NH_4^+ \cdot L^{-1}$, which was maintained for a long period of time (from the beginning of the study until day 150) until MLSSs and the SRT

were increased (Figure 5.8, bottom). Phosphorus in the effluent also rose to 3-4 mg $P\text{-}PO_4^{3-}\cdot L^{-1}$, although it was completely removed in a shorter period of time than for ammonium (100 days, Figure 5.10, bottom). As can be seen in Figure 5.10, when nitrification was restored at day 150, an average of 2.3 mg $N\text{-}NO_2^-$ appeared in the effluent due to nitritation (ammonium oxidizing bacteria (AOB) oxidizes ammonium to nitrite). It was not until the system was able to develop nitrite oxidizing bacteria (NOB) at day 180 that nitratation took place (nitrite oxidation to nitrate) and 5.3 mg $N\text{-}NO_3^-\cdot L^{-1}$ appeared in the effluent with nitrite being reduced to 0.7 mg $N\text{-}NO_2^-\cdot L^{-1}$.

According to the nitrogen balance (theoretical NO_x^-, see section 3.4.4), a mean concentration of 9 mg $N\text{-}NO_3^-\cdot L^{-1}$ should have been found in the effluent due to the nitrification of the ammonium fed in the second feeding period of the cycle. This concentration was not observed throughout the study (Figure 5.10, bottom). This means that simultaneous nitrification-denitrification (SND) was occurring in the process when nitrite or nitrate were produced by nitrification. In order to show the effect of SND during the study, Figure 5.11 presents the theoretical and experimental values of NO_x^-, as a sum of NO_2^- and NO_3^-. The theoretical concentration was calculated as the complete oxidation of the ammonium added in the second feed event, supposing complete denitrification in the previous anoxic phase. If the difference between experimental and theoretical values presents positive values, NO_x^- is accumulated in the reactor, while when it is negative, SND is performed (see section 3.4.4).

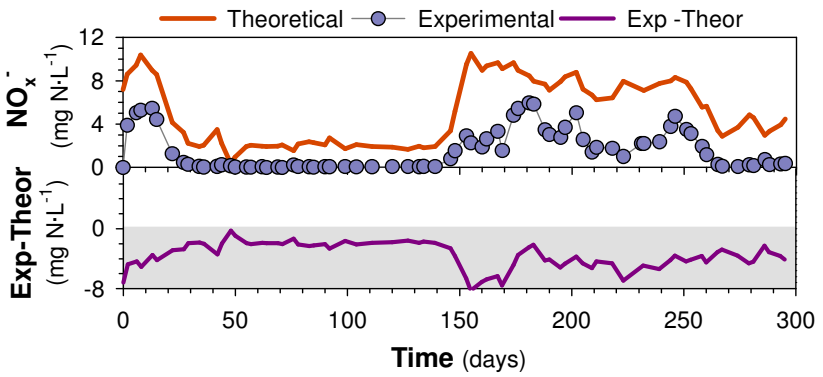

Figure 5.11. Theoretical and experimental nitrate and nitrite (NO_x^-) effluent concentrations (top) and nitrate and nitrite removed by SND (experimental – theoretical) (bottom). Shadowed area corresponds to SND performance.

The data shows that SND dominated throughout the granular reactor, even more when complete nitrification was achieved. Experimental influent and effluent evaluation of nitrogen and phosphorus provided an overview of BNR processes inside the reactor. However, the effect of nitrite, nitrate and SND in granular sludge during the cycle performance was an unknown factor. Because of that, cycle studies were carried out periodically to evaluate nutrient removal performance under these conditions. Figure 5.12 shows a typical cycle profiles when nitrite was the sole product of nitrification and when nitrate was obtained in the effluent.

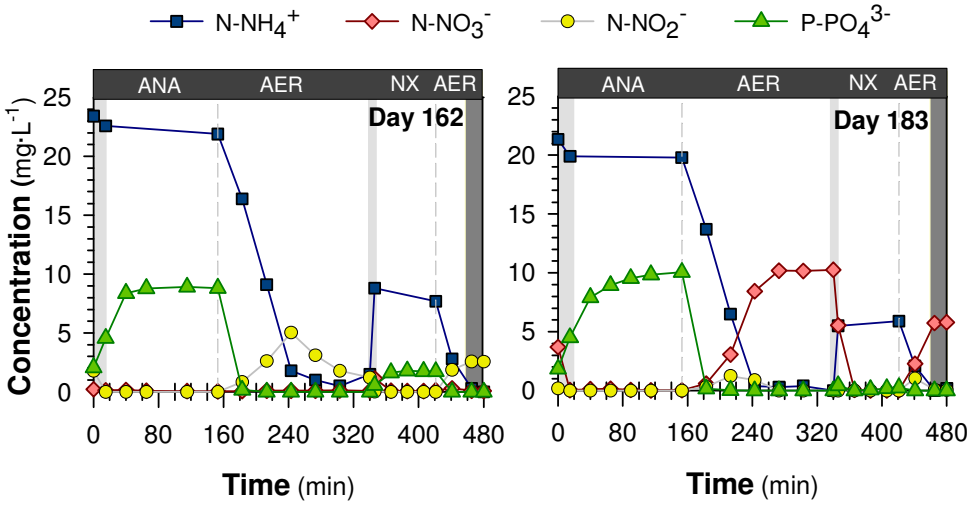

Figure 5.12. Cycle profile concentrations of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) at day 162 (nitrite production, left) and day 183 (nitrate production, right). ANA, AER and NX stand for anaerobic, aerobic and anoxic phases.

During the anaerobic phase, phosphorus accumulating organisms (PAOs) took up the organic matter while phosphate was being released into the bulk liquid and stabilized the phosphorus concentration at 8.9 and 10 mg $P\text{-}PO_4^{3-}\cdot L^{-1}$ before minute 90 at day 162 and 183, respectively (Figure 5.12). In the subsequent aerobic phase, the phosphate was totally taken up in 30 minutes in both cycles.

When nitrite was the main product of nitrification (Figure 5.12, left), ammonium consumption (20.1 mg $N\text{-}NH_4^+\cdot L^{-1}$) at minute 240 of the aerobic phase was unbalanced with nitrite production (5.1 mg $N\text{-}NO_2^-\cdot L^{-1}$). This indicates

that SND had taken place under aerobic conditions. Nitrite did not appear in the bulk liquid until all the phosphate had practically been removed (minute 180). Furthermore, nitrite concentration decreased after the available ammonium had been completely oxidized, so that at the end of the first aerobic phase (minute 340) only 1.2 mg $N\text{-}NO_2^-\cdot L^{-1}$ were present in the media. The maximum total SND calculated (see section 3.3.5) was 65%. After the second feeding (minute 340), the residual nitrogen from the previous aerobic phase was rapidly denitrified. As no nitrite or nitrate was present in the system, anaerobic conditions were achieved and phosphate was released using the surplus organic matter supplied in the second feed. In the last aerobic phase, ammonium, phosphate and some nitrite were removed and a maximum SND of about 33% was achieved.

When nitrate rather than nitrite was produced while 19.8 mg $N\text{-}NH_4^+\cdot L^{-1}$ were being nitrified (Figure 5.12, right), 10.3 mg $N\text{-}NO_3^-\cdot L^{-1}$ accumulated at the end of the first aerobic phase. Nitrate only appeared after phosphate had been totally removed from the system (minute 180). In this case, maximum SND reached 29%. After the second feed, denitrification of the remaining nitrate took place and no phosphorus was released, therefore, no phosphorus activity was detected during the last aerobic phase. Furthermore, all the ammonium was completely oxidized to nitrate and no SND activity was observed.

When the two cycle studies are compared, it can be observed that higher SND was obtained when nitrite was the final product of nitrification. This can be seen from Figure 5.11, where more nitrogen was removed by SND when nitrite appeared in the effluent (between day 150 and 180). Furthermore, both cycle studies (Figure 5.12) evidenced nitrogen and phosphorus removal. The fact that neither nitrate nor nitrite was released in the media until phosphorus was depleted to zero seems to suggest the occurrence of aerobic nitrification coupled with denitrification and phosphorus removal. This process has been named simultaneous nitrification, denitrification and phosphorus removal (SNDPR) (Zeng *et al.*, 2003c) and is based on the capacity of some PAOs to denitrify (denitrifying PAOs or DPAOs). The biggest differences in the cycle studies of both periods were found while nitrification and phosphate uptake were taking place in the first aerobic phase. The highest maximum SND was

achieved using nitrite as the electron acceptor, reaching 65% in the first aerobic phase while phosphate was being removed (Figure 5.12, left). When nitrate was the main nitrification product (Figure 5.12, right) SND was also observed in the first aerobic phase and neither SND nor phosphorus removal was detected in the second phase. This shows that SND could be enhanced when coupled with the EBPR process. However, because of the fast phosphorus removal, data from these experiments suggested a link between the two processes, but it was not conclusive for DPAO activity.

5.4.2.2 Influence of nitrification products on simultaneous nitrogen and phosphorus removal

Results from the granular SBR's evolution indicated different behaviors depending on the product of nitrification released into the media (either nitrite or nitrate). For this reason, batch tests were carried out at day 190 of the operational period to evaluate SNDPR activity in the granular sludge using different electron acceptors (i.e. nitrite, nitrate and oxygen). Four batch tests were performed under anaerobic-anoxic conditions using both nitrate and nitrite and anaerobic-aerobic conditions at different dissolved oxygen concentrations (see section 5.3.2). Figure 5.13 depicts the phosphate and nitrogen profiles obtained during the experiment. Results are presented as specific concentrations so the data from each test can be compared.

During the first part of the test, phosphate was released until stabilization (after 60 minutes) due to organic matter uptake by PAOs (around 40 mg $C \cdot L^{-1}$). In the anaerobic-anoxic test with nitrate (Figure 5.13A), phosphorus was completely taken up while nitrogen was reduced from 20.7 to 5.0 mg $N\text{-}NO_3^- \cdot L^{-1}$. The remaining nitrogen was reduced under anoxic conditions by denitrifying organisms other than DPAOs, as observed in the pilot plant. In the case of the nitrite batch test (Figure 5.13B), phosphorus and nitrogen where simultaneously removed until minute 240. At this point there was a nitrite concentration of 4.4 mg $N\text{-}NO_2^- \cdot L^{-1}$ and phosphorus was stabilized at 5.6 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$. The phosphate concentration remained the same until the end of the experiment, which showed that nitrite had been completely reduced by non DPAO organisms.

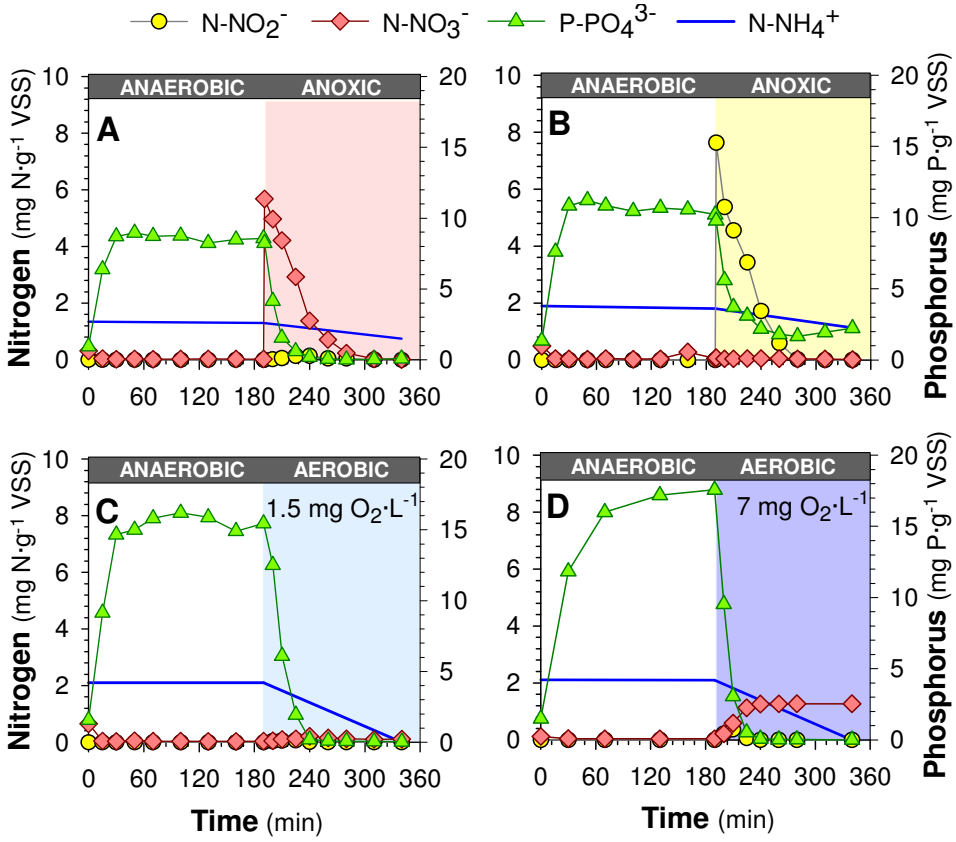

Figure 5.13. Batch tests for simultaneous nitrogen and phosphorus removal. Ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) specific profiles under anoxic conditions using nitrate (A), anoxic conditions using nitrite (B), low oxygen concentration (1.5 mg $O_2 \cdot L^{-1}$) (C), and high oxygen concentration (7.0 mg $O_2 \cdot L^{-1}$) (D).

As previously noted in other experiments (see section 4.4.2) free nitrous acid (FNA) can completely inhibit the phosphate uptake rate (PUR). However, even at the beginning of the anoxic phase when nitrate was pulsed, a maximum of 0.0053 mg $N\text{-}HNO_2 \cdot L^{-1}$ was obtained while phosphate was being taken up. This means that the end of phosphate removal in Test B was not due to FNA inhibition, as values were reduced at this point to 0.0004 mg $N\text{-}HNO_2 \cdot L^{-1}$. Both anoxic experiments showed a smooth decrease of 0.6 mg $N\text{-}NH_4^+ \cdot L^{-1}$, attributable to the biomass assimilation process.

Aerobic tests (Figure 5.12 C and D) performed similarly in terms of phosphate removal, which was all taken up at minute 240 in both reactors. Oxygen worked as an electron acceptor for phosphorus removal, but also provided nitrification conditions, with the result that a small amount of ammonium was completely removed from both systems. However, nitrate was only detected when 7 mg $O_2 \cdot L^{-1}$ were supplied in Test D, when a final concentration of 3.0 mg $N\text{-}NO_3^- \cdot L^{-1}$ was obtained from the nitrification of 4.8 mg $N\text{-}NH_4^+ \cdot L^{-1}$ fed in the experiment. This shows that lower oxygen concentrations enhanced the SNDPR process that took place with the granules (mean size of 1 mm) obtained from SBR-1. Table 5.5 summarizes the parameters related to phosphate obtained during the batch test experiments.

Table 5.5. Phosphorus ratios during batch test experiments: phosphate uptake rate (PUR) and anoxic PUR-aerobic PUR ratio (PURanox/PURaer).

Test	Electron acceptor	PUR mg $P \cdot g^{-1}VSSh^{-1}$	PURanox/PURaer [mg $P\text{-}PO_4^{3-} \cdot L^{-1}]_0$
A	Nitrate	13.64	0.47
B	Nitrite	12.20	0.42
C	Oxygen (1.5 mg $O_2 \cdot L^{-1}$)	23.14	-
D	Oxygen (7 mg $O_2 \cdot L^{-1}$)	29.26	-

The PUR obtained from anaerobic-aerobic tests showed higher values than those obtained from anaerobic-anoxic tests. This is explained by the fact that DPAOs are one group of the overall PAO population with enzymes that allow them to accomplish anoxic EBPR metabolism (Zeng *et al.*, 2003a). On the other hand, anaerobic-anoxic tests resulted in similar PUR values when using nitrate and nitrite as electron acceptors (Table 5.5), as well as similar ratios for DPAO activity (47% in the nitrate test, 42% in the nitrite test). Therefore, nearly half of the PAO population was able to remove phosphorus under anoxic conditions. Finally, in order to show DPAO activity using different electron acceptors, phosphorus and nitrogen concentrations are given in Figure 5.14.

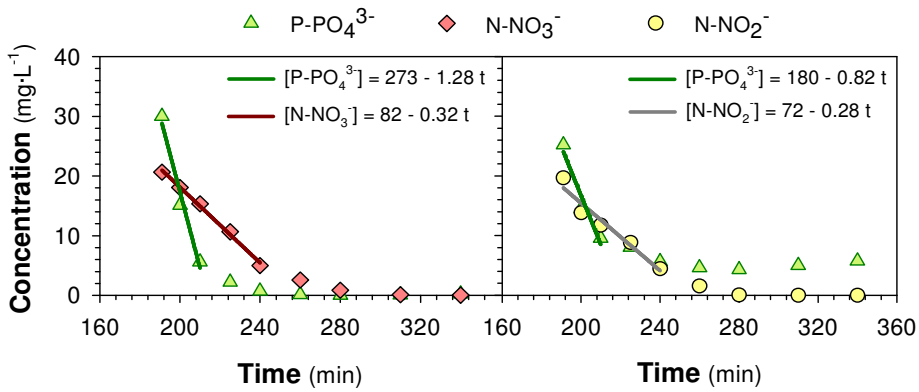

Figure 5.14. Phosphate (PO_4^{3-}) and nitrate (NO_3^-) (left) and nitrite (NO_2^-) (right) concentrations under anoxic conditions. Regressions lines show the removal rates for each compound.

In terms of phosphorus uptake under anoxic conditions, the P_{UP}/N_{REM} and P_{UP}/COD ratios were calculated and summarized in Table 5.6. P_{UP}/N_{REM} was calculated from the relation of the slopes of the regression lines of Figure 5.14. P_{UP}/COD was found with the ratios of organic matter requirements for denitrification from the literature (1.71 g COD·g^{-1}N-NO_2^-; 2.86 g COD·g^{-1} N-NO_3^-; Tchobanoglous *et al*., 2003).

Table 5.6. Phosphorus ratios during batch test experiments: phosphate uptake per nitrogen removed (P_{UP}/N_{REM}) and per organic matter consumed (P_{UP}/COD).

Test	Electron acceptor	P_{UP}/N_{REM} mg P·mg^{-1} N	P_{UP}/COD mg P·mg^{-1} COD
A	Nitrate	4.0	1.4
B	Nitrite	2.9	1.7

According to the values obtained, a higher amount of phosphate was removed using nitrate (4.0 mg P·mg^{-1} N) rather than nitrite (2.9 g P·g^{-1} N) as the electron acceptor. With regards to the organic matter requirements, nitrite as electron acceptor would be slightly more efficient with the same amount of COD (1.7 mg P·mg^{-1} COD) in order to remove phosphorus by DPAO organisms. Therefore, nitrate may be used when high amounts of phosphorus have to be removed while nitrite would enhance phosphorus removal when organic matter limitations are affecting the system. However, all these values were obtained from theoretical denitrification organic matter requirements, which could be

different from the stoichiometry applied in DPAOs as they denitrify using internally stored poly-β-hydroxyalkanoates (PHA). In this connection, the role of internal polymer limitation as the responsible for DPAO activity, such as glycogen or PHA, should be investigated.

5.4.3 Microbiological analyses: FISH and SEM

Microbiological analyses complement an engineering perspective and usually help to a better understanding of biological processes. Fluorescence in situ hybridization (FISH) was used throughout the study to detect the presence of microorganisms performing nitrogen and phosphorus removal. Figure 5.15 depicts the evolution of nitrifiers (ammonium and nitrite oxidizing bacteria, AOB and NOB, respectively) during the study.

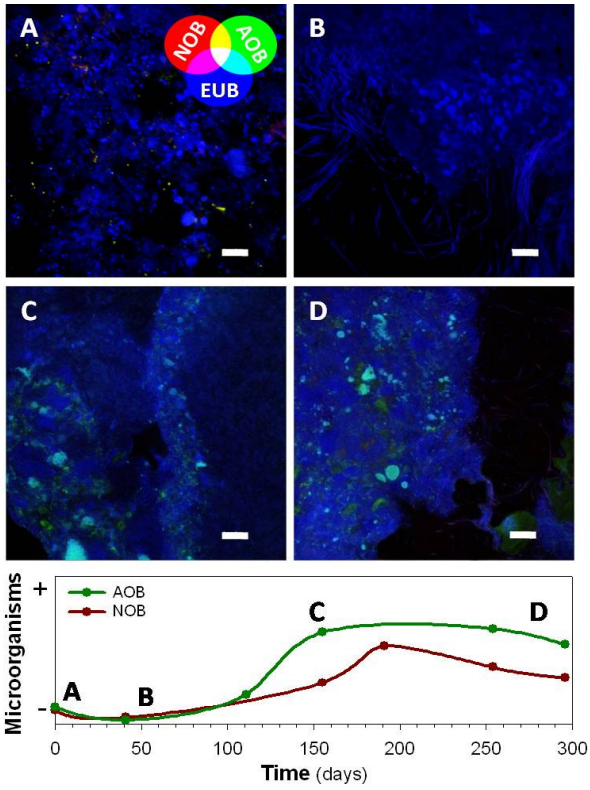

Figure 5.15. Confocal laser micrographs (top) for ammonium oxidizing bacteria (AOB, in green), nitrite oxidizing bacteria (NOB, in red) and *Eubacteria* representing for all bacteria (EUB, in blue), and their qualitative evolution (bottom) throughout granular performance. Scale bars 20 µm.

The qualitative graph and the FISH micrographs show that AOB and NOB were present with very low concentrations at the beginning of the study. During the granulation period (from day 0 to day 20), nitrification was lost (see section 5.4.2.1), and therefore the population was severely reduced. AOB started to increase before NOB, because of that, some nitrite was observed in the effluent when nitrification was recovered (Figure 5.10). Afterwards, NOB population increased, but the percentage was always lower than with the AOB microorganisms. This could have been caused by SND taking place in the granules, which would reduce the availability of substrates (nitrite) for NOB. Nitrifying bacteria was maintained in the same proportion until the end of the study, when FISH quantification could be carried out. The percentages obtained at the end of the study were 9% for AOB and 6% for NOB.

Because AOB and NOB are aerobic bacteria, it was postulated that they would be distributed in the outer parts of the granules as oxygen is diffused into the core of the particles, thereby reducing its concentration. For this reason, FISH was used to scan through a granule, from the outer part (z = 0 µm) to the inner part of the particle (z = 22 µm). Figure 5.16 shows the results obtained with the confocal laser scanning microscope (CLSM) at day 20 of the study when the first granules appeared.

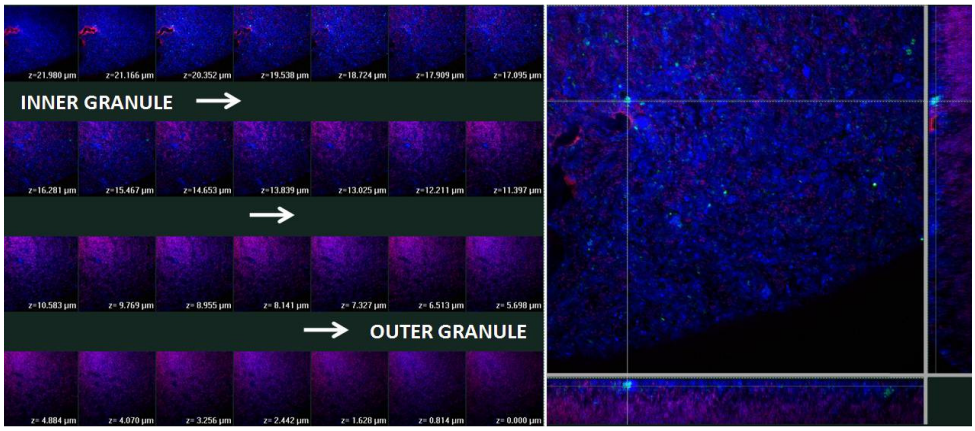

Figure 5.16. Confocal laser micrographs for ammonium oxidizing bacteria (AOB, in green), nitrite oxidizing bacteria (NOB, in red) and *Eubacteria* representing for all bacteria (EUB, in blue). Sequences from a granule on the Z axis (left) and compilation of images (sum of all images) obtained (right) at day 20 of the study.

Nitrifying bacteria were detected all over the granule as the reddish color from the CLSM micrographs shows (Figure 5.16, left). In the inner part of the granule (z = 22 µm) only a few NOB were detected, while the major concentration was found in the particle's surroundings. From the compilation of images obtained after the scan through the z axis, some AOB (green color) were also observed (Figure 5.16, right). AOB were found at 11 µm beneath the surface of the aggregate, which suggest that oxygen diffusion through the particles, which is reduced when it gets deeper into the core, affects the nitrifying bacteria distribution. AOB and NOB were detected at the outer part of the granule within 15 µm of the surface.

With regard to the EBPR population, FISH analysis was carried out to detect PAOs as *Accumulibacter* and their glycogen accumulating bacteria (GAOs) competitors as *Competibacter*. Results of CLSM micrographs and qualitative evolution are presented in Figure 5.17.

At the beginning of the study, as happened with nitrifying bacteria, the EBPR population was reduced due to a biomass washout resulting from the granulation strategy (see section 5.4.1.1), which decreased the SRT and impaired the phosphorus removal performance. Once phosphorus removal started to recover from day 50 to day 150, the biomass was enriched in terms of PAOs, even though GAOs also increased by a lesser quantity. From day 150 to 200, PAOs decreased sharply while GAOs remained at similar concentrations. This coincided with the appearance of nitrite in the effluent of the reactor (Figure 5.10) which was suggested to inhibit aerobic phosphorus uptake (Saito *et al*., 2004; Oehmen *et al*., 2007) and caused organic matter competition in the subsequent anaerobic phase. Even though batch experiments suggested that granular sludge activity was not as much affected to FNA concentrations as in the un-adapted floccular sludge from the literature, PAOs growth could be progressively decreased. However, GAOs were not as much affected as PAOs by nitrite during this period. According to Ye *et al.* (2010) GAO metabolism was not reduced until 0.0015 mg $N\text{-}HNO_2 \cdot L^{-1}$ were reached in an un-adapted nitrite reactor, while PAOs were completely inhibited at 0.0010 mg $N\text{-}HNO_2 \cdot L^{-1}$. Furthermore, Pijuan *et al.* (2011) stated that FNA presence will likely provide

competitive advantage to GAOs over PAOs. This could be related to the metabolism of *Competibacter* bacteria, which obtain their energy only from glycogen degradation and not from polyphosphate as PAOs do. The effect of nitrite on GAO organisms needs to be studied in more depth in the future.

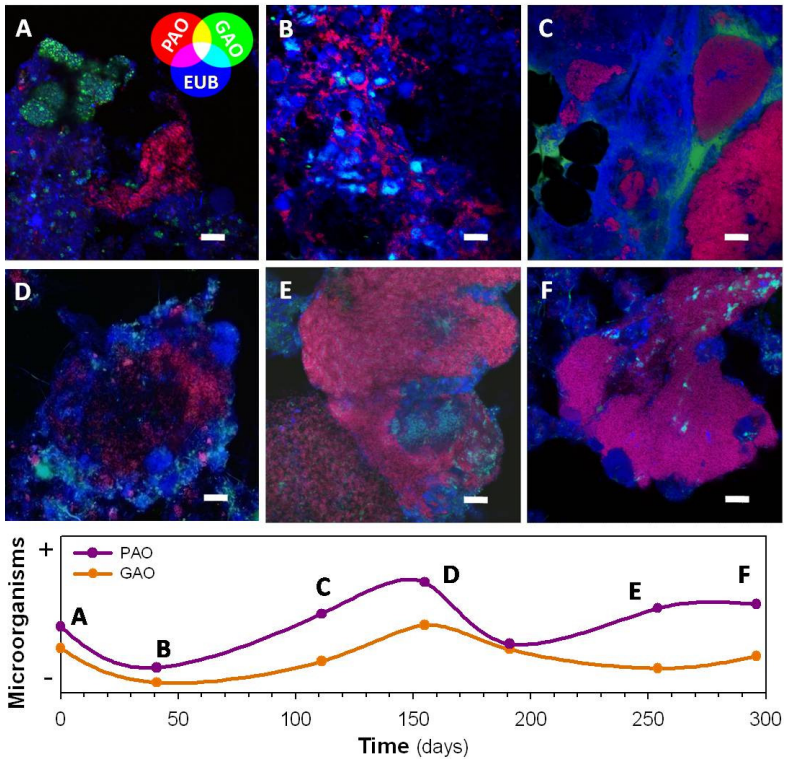

Figure 5.17. Confocal laser micrographs (top) for phosphate accumulating organisms (PAOs, in red), glycogen accumulating organisms (GAOs, in green) and *Eubacteria* representing for all bacteria (EUB, in blue), and their qualitative evolution (bottom) throughout the granular performance. Scale bars 20 µm.

When nitrite production was reduced, the phosphorus removal population was enriched ahead of the total bacteria while GAOs remained at the same concentration. At the end of the study when FISH quantification was carried out, 58 % of *Accumulibacter* and 18 % of *Competibacter* were achieved. A part from the enrichment of PAOs over the course of the study, what can be seen from the CLSM micrographs is that PAOs tend to form big colonies of bacteria while GAOs are found in smaller groups of microorganisms spread all around the biomass sample.

Microbiological analyses were used to identify the microbial composition of the granules. However, to study the structure of the particles that developed, scanning electron microscopy (SEM) was also applied to some granules. Figure 5.18 depicts the images obtained from this analysis with an example of granules from SBR-1 when the system was stable at the end of the study (day 300).

In accordance with the results of the physical and morphological study of granular sludge for different granulation strategies (see section 5.4.1.2), there were some filamentous bacteria in SBR-1 surrounding the particles which did not affect the settling properties of the system. SEM micrographs (Figure 5.18 A and B) also show that the walls of the granules were completely surrounded by filaments. When the outer parts of the particles (Figure 5.18 C) are looked at more closely, filamentous bacteria seem to be supporting other bacteria, which would suggest filaments help to make the granules more compact.

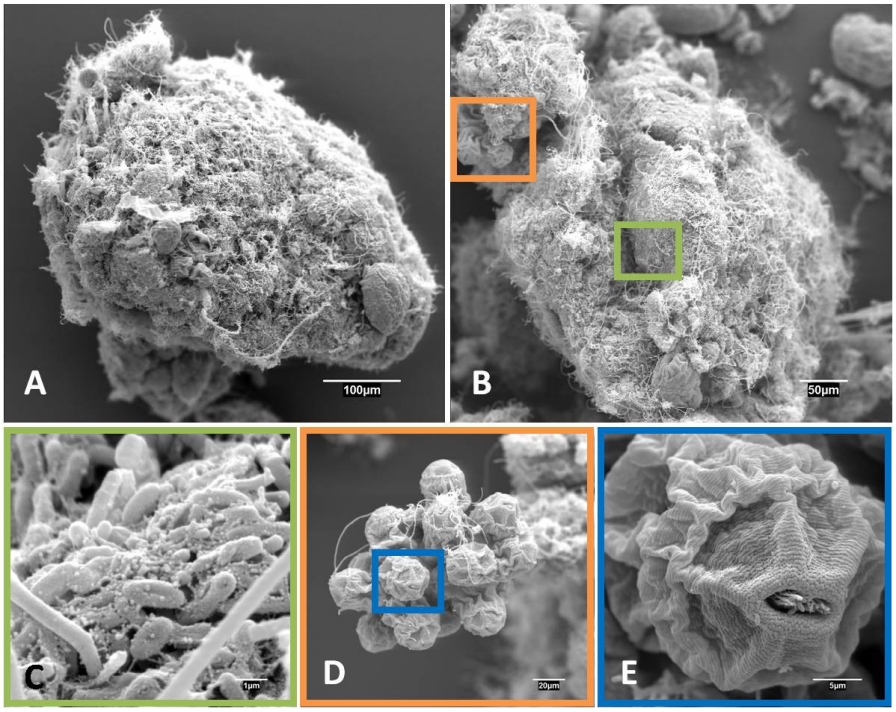

Figure 5.18. Scanning electron microscope images from different granules from SBR-1 (A & B) and zoom images from granule B (C, D, E).

Apart from filamentous bacteria, other protozoa were detected attached to the surface of the granules. In Figure 5.18D, groups of *vorticella*-like organisms were detected in a dehydrated condition after SEM treatment. The presence of this kind of organisms would enhance organic matter removal as they filter the nutrients from the water through the ciliates at the top of the organisms (Figure 5.18E). This would result in protozoa attached to granules helping with organic matter and nutrient removal from wastewater.

5.5 Discussion

5.5.1 Effect of VER and settling time decrease in granulation with low-strength wastewater

The first consequence of the selection of particles by decreasing the settling time was a biomass washout independent of the VER applied (40%, 50% or 60%). The main difference when different volumes were used in the SBR, which increased the organic loading rate (OLR) proportionally, was the recovery of MLSS concentration and the size and shape of granules obtained. At the lowest VER of 40%, the granules reached by far the biggest mean diameter and highest biomass concentration in all three reactors.

In spite of enhanced granulation when the VER was increased to 50%, as might have been expected because of the increase in OLR, biomass growth slowed down due to an increase in treated water discharged and, consequently, a higher washout of particles. As a result, mean size and MLSS concentration had lower values than in the 40% VER reactor. This situation was aggravated when the VER was raised to 60% VER, at which level the granules could not properly develop. Furthermore, when the OLR was increased and biomass concentration was not improved, more organic matter was available under aerobic conditions and an outgrowth of filamentous bacteria appeared at the end of the study in the 50% and 60% VER systems. In this study, a filamentous net entrapped the granules, making the settling performance and the continuation of the experiment difficult.

5.5.2 Effect of granulation on nutrient removal

The gradual reduction in settling time caused a considerable decrease in biomass concentration, and as a consequence SRT fell to values below the minimum retention time for nitrifying bacteria growth. With granular formation, MLSS started to increase and phosphorus removal was the first process to recover. Nitrogen removal was only reestablished when biomass washout diminished and SRT rose to values sufficient for AOB to grow on the particles' surface. As a consequence of lack of substrate (nitrite), NOB community development was slower and some nitrite appeared in the effluent. The cause of this longer time to achieve nitratation (oxidation of nitrite to nitrate), compared with the beginning of nitrification in conventional floccular sludge, could have been due to the presence of granules enhancing the SND process, either using nitrite or nitrate. Nitrite presence in the system could also have affected EBPR performance. Although phosphorus removal efficiencies did not decrease, the microbial population (PAO) was reduced after the biomass was exposed to nitrite, and consequently free nitrous acid (FNA), concentrations. The maximum FNA obtained in the reactor was 0.0062 mg N-$HNO_2 \cdot L^{-1}$ while reductions in PAO activity from un-adapted floccular sludge reactors were reported at 0.002 mg N-$HNO_2 \cdot L^{-1}$ (Saito *et al.*, 2004, Zhou *et al.*, 2007). Furthermore Pijuan *et al*. (2011) found that FNA of over 0.007 mg N-$HNO_2 \cdot L^{-1}$ completely inhibited PAOs and GAOs growth. However, at lower concentrations PAOs growth was reduced more significantly compared to GAOs. Then, either inhibition or organic matter competition can be the cause of a PAO population reduction, and there needs to be sufficient SND in granular systems to avoid nitrite in the reactor.

Granular performance in terms of MLSSs and size was dynamic during the study. With regard to biomass concentrations, the cycle profiles showed that immediately after solids inside the reactor reached a maximum point, granules were disrupted and the smallest particles were washed out through the effluent, causing a decrease in MLSSs. This cyclical behavior was proportionally related to the SRT, which also affected the nitrogen removal performance when SRT was too low, although phosphorus removal was maintained.

Treating low-strength wastewater implies working at a low OLR, which results in a insufficient biomass increase in time as reported in de Kreuk and van Loosdrecht (2006). In this study, the low MLSS and the dynamic performance of granular sludge treating domestic wastewater caused some instability, mainly in the nitrogen removal process, although organic matter and phosphorus removal remained stable throughout. However, after a long start-up period, once big particles dominate the reactor, complete BNR can be achieved. Granulation in low-strength wastewater is possible, but further research needs to be carried out to overcome the bottleneck in the process, which is the instability of the system while a fully granular reactor is in the process of being obtained.

5.5.3 Effect of nitrite and nitrate compounds on simultaneous nitrogen and phosphorus removal in granular sludge

The SBR cycle studies involved simultaneous nitrogen and phosphorus removal. According to the literature, DPAOs in BNR systems are highly beneficial in terms of having a lower COD requirement (because the same carbon source is used for both N and P removal) and a reduced aeration cost (Lemaire *et al*., 2008b). However, reliable termination of nitrification at nitrite (nitritation) has proved difficult to achieve in the treatment of domestic wastewater (Blackburne *et al*., 2008). The application of granular sludge could be helpful for SNDPR using nitrite, evidenced by the fact that the nitratation process was seen to take longer in this study compared to when floccular sludge was used.

Results obtained from the batch tests showed that PAOs used both nitrite and nitrate for phosphorus removal in granular sludge, with similar DPAO activity. However, a P_{UP}/N_{REM} ratio was found that was higher when using nitrate than nitrite as the electron acceptor. This could be because of the higher electron demand from nitrate (5 electrons) compared to nitrite (3 electrons) during denitrification. When the requirement of organic matter for denitrification was taken into account (Tchobanoglous *et al*., 2003), nitrite was found to be more effective for phosphate removal when organic matter limitations affect the system. So, the use of nitrate or nitrite should be evaluated according to the system requirements.

Although nitrite leads to an enhancement of SNDPR, it has been considered as a potential inhibitory compound for P uptake (Meinhold *et al*., 1999; Saito *et al*., 2004). However, Zhou *et al*. (2007 & 2008) suggested nitrous acid rather than nitrite is the true inhibitor in the phosphorus uptake (0.02 mg $N\text{-}HNO_2 \cdot L^{-1}$ completely blocks phosphate uptake). When dealing with low-strength wastewater, progressive ammonium oxidation and SND makes difficult to obtain high amounts of nitrite. Therefore, even if nitrite is formed in granular sludge, FNA may not be high enough to inhibit phosphorus removal, as shown by the fact that the PUR was not affected in this study.

5.6 Conclusions

Granulation treating low-strength wastewater (i.e., domestic sewage) has been proven to be effective applying short settling times although loading rates lower than 1 Kg $COD \cdot m^{-3} d^{-1}$ are applied. However, applying higher VERs in this study order to obtain higher loading rates of 50% or 60% rather than 40%, led to granule instability due to filamentous bulking. Furthermore, bringing about granulation by increasing the VER induced the presence of more homogenous but smaller particles, causing a major washout of biomass.

In terms of nutrient removal, granular sludge cultivated with low-strength wastewater was able to treat organic matter with efficiencies of around 86%. Nitrogen and phosphorus removal were unstable during granulation due to poor SRT and MLSS concentrations. Once these parameters were recovered, complete BNR was achieved. This shows that phosphorus and particularly nitrogen removal performance are closely linked to the granular stabilization of a system, which requires a longer start-up period when treating low-strength wastewater.

Finally, granular sludge allows interactions between different microorganisms, and PAOs can take advantage of this using both nitrite and nitrate as products of nitrification under aerobic conditions because of oxygen diffusion into the particles. Both electron acceptors (nitrite and nitrate) have similar rates for phosphate uptake. However, nitrite would enhance SNDPR efficiency as it has a lower organic matter requirement per unit of phosphate uptake. Furthermore,

nitrite would not cause inhibition in phosphate uptake rates as granular sludge can diffuse its concentration and increase the threshold inhibition levels, which will be hardly achieved with low-strength wastewater.

Chapter 6. Granulation in real domestic wastewater for nutrient removal: influence of primary treatment

Granular reactors have been developed when treating synthetic and high-strength wastewater, but few studies have been done on reactors treating municipal wastewater, and little research has been carried out to study nutrient removal in granules with low-strength wastewater. The aim of this chapter is to investigate the development of granules with domestic wastewater. Raw and settled wastewater are compared for both granulation and nutrient removal to see if particles from raw wastewater enhance granular formation.

This chapter is based on the following publications:

Coma, M., Puig, S., Barceló, M., Balaguer, M.D., Colprim, J. 2010. Influence of primary treatment on nutrient removal from domestic wastewater: moving to granular sludge. *Sustainable solutions for small water and wastewater treatment systems (S2Small2010)*, (19-22 April, Girona, Spain), Oral.

Coma, M., Puig, S., Oehmen, A., Carvalho, G., Colprim, J., Balaguer, M.D. 2011. Aerobic granular development and nutrient removal enhanced by raw domestic wastewater. *In preparation*.

6.1 Introduction

The performance of aerobic granular sludge systems has been assessed in laboratory-scale reactors treating synthetic (Chapter 5) and, more recently, industrial wastewater, such as that from dairy and livestock production (de Kreuk and van Loosdrecht, 2006; Lemaire, 2007; Yilmaz *et al.,* 2008). The few studies of municipal wastewater treatment that have used aerobic granules are referenced below. Some studies carried out have involved treating synthetic influents with concentrations similar to those of low-strength wastewater for nutrient removal and granulation purposes (see section 5.4.2; Li *et al*., 2007). Others evaluated nutrient removal performance in domestic wastewater using granules formed with acetate (Liu *et al*., 2007; Wang *et al*., 2009). Granular development with low-strength wastewater (such as domestic wastewater) has been found to be unsuitable for organic loads lower than 1 Kg COD·$m^{-3}d^{-1}$ and to result in long start-up periods (de Kreuk and van Loosdrecht, 2006). There have been studies of chemical oxygen demand (COD) and nitrogen removal treating domestic wastewater with higher loads of 3 Kg COD·$m^{-3}d^{-1}$ (Liu *et al.*, 2010) or performing granulation in packed SBR systems (Ramadori *et al*., 2006; Di Iaconi *et al*., 2008). Ni *et al*. (2009) reported pilot-scale granulation for organic matter and ammonium treatment at 0.6-1.0 Kg COD·$m^{-3}d^{-1}$, but without either denitrification or phosphorus removal.

Primary treatment provides readily biodegradable organic matter as the result of pre-fermentation, but sometimes it is not viable because of the requirement for large surface areas. Septic tanks often have to be installed to meet the system's needs, but this increases treatment costs (Geenens and Thoeye, 2000). The need for directly treating raw wastewater results in low concentrations of readily biodegradable organic matter and high amounts of particulate matter. The particulate fraction of the wastewater does not enhance nutrient removal (Puig *et al.*, 2010), but can provide support material to improve granulation. According to Tsuneda *et al*. (2004) granules are formed when biomass grow around a core of Fe precipitation. In other studies, crushed granules mixed with flocs have been used as seed sludge for granular reactors (de Kreuk *et al*., 2005). Pijuan *et al*. (submitted) have even demonstrated that adding crushed granules to the seeding sludge significantly reduces the start-up

time in aerobic granular reactors when dealing with nutrient rich wastewater (i.e., abattoir wastewater). It therefore appears that, inorganic or even organic particles coming from raw wastewater might be a starting point for granulation performance.

6.2 Objectives

The purpose of this work was to study the influence of raw wastewater fed into a granular sludge system on domestic wastewater treatment. The challenges presented by the use of particulate and decanted low-strength wastewater were compared and evaluated for both granulation and nutrient removal performance.

6.3 Experimental procedure

Three lab-scale sequencing batch reactors (SBRs) (see section 3.1.1) were used in this study. They were seeded with sludge from the Sils-Vidreres wastewater treatment plant (WWTP) (Girona, Spain) and fed with real domestic wastewater collected from the Quart WWTP (1000 population equivalent (PE), Girona, Spain). SBR-1, -2 and -3 were fed, respectively, with raw wastewater, decanted wastewater to remove particles from the wastewater and decanted wastewater dosed with ethanol. Ethanol was used as external carbon source in order to increase the organic loading rate (OLR) to values similar to the raw influent (Puig *et al.*, 2007a). Table 6.1 summarizes the composition characterization of these streams.

The reactors were operated in eight-hour cycles with two feeding events (70% of the volume in the first feed) followed by two sequences of anaerobic-aerobic and anoxic-aerobic phases, respectively, as shown in Figure 6.1. The first part of the cycle focused on enhanced biological phosphorus removal (EBPR). The second part was based on nitrogen removal. The anoxic phase before the second feed was used to decrease the oxygen concentration in the reactor and prevent aerobic organic matter removal when the influent was introduced. The wastewater provided the organic matter for denitrification. The cycle ended

with an aerobic phase to oxidize the ammonium supplied in the second filling period, a settling period and decant or drawing phases.

Table 6.1. Wastewater composition characterization used in this study.

	SBR-1 Particulate WW	SBR-2 Decanted WW	SBR-3 Decanted WW + EtOH	Units
COD_T	474 ± 21	172 ± 6	281 ± 6	mg COD·L^{-1}
COD_s	161 ± 8	73 ±6	174 ± 8	mg COD ·L^{-1}
BOD_5	254 ± 3	78 ± 12	183 ± 4	mg COD ·L^{-1}
TN	83 ± 1	46± 1	45 ± 1	mg N·L^{-1}
NH_4^+	66 ± 2	38 ± 1	35 ± 1	mg N·L^{-1}
TP	8 ± 1	5± 2	4 ±1	mg P·L^{-1}
PO_4^{3-}	6.17 ± 0.06	3.87 ± 0.02	1.99 ± 0.02	mg P·L^{-1}
TSS	166	65	63	mg·L^{-1}
VSS	149	65	63	mg·L^{-1}
COD_T/N/P	100:15:1.3	100:23:2.0	100:14:0.9	g COD:g N: g P

The three SBRs were run for 50 days (from day -50 to day 0) with a 25-minute settling time to acclimate the sludge to nitrogen and phosphorus removal. At day 0, the settling time was progressively decreased to 2 minutes for granulation purposes. The hydraulic retention time (HRT) and volume exchange ratio (VER) were fixed at 0.7 days and 48% for the entire study. These conditions were selected in accordance with the results reported in Chapter 5, where a VER between 40-50% allowed both a higher OLR and granulation. The sludge residence time (SRT) was not controlled as the sludge was wasted through the effluent when the settling time was decreased.

Figure 6.1. 8-h cycle applied in the SBRs for nutrient removal purposes.

6.4 Results

6.4.1 Granulation with raw and settled wastewater

The three SBRs were run for 50 days with 25 minutes of settling time treating raw wastewater (SBR-1), decanted or settled wastewater (SBR-2), and decanted wastewater but dosed with ethanol (SBR-3). After the acclimation period, the settling time was gradually decreased from 25 to 2 minutes so that the heavier sludge flocs could be selected and granulation enhanced. The settling time reduction period was longer than the one proposed for synthetic wastewater (see section 5.4.1.1), lasting 40 days for SBR-1 and SBR-2 and 45 days for SBR-3. This change was proposed to reduce the loss of biomass during settling time reduction. Figure 6.2 shows the settling time reduction applied in the reactors under study.

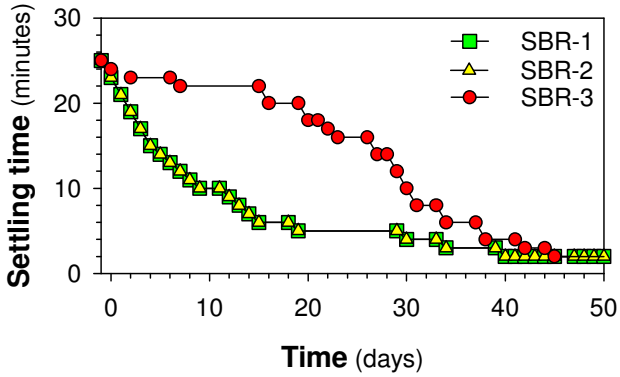

Figure 6.2. Settling time reduction applied in SBR-1 (raw WW), SBR-2 (decanted WW) and SBR-3 (decanted + ethanol WW).

SBR-1 and -2 were inoculated at the same time and had the same settling time reduction profile. SBR-3 was started after SBR-2 and a more conservative reduction (Figure 6.2) with a smoother decrease at the beginning and a faster one at the end was applied. The SBR-3 strategy was based on experimental observation; the settling time was set to correspond to a minimum layer of sludge to avoid biomass washout through the effluent and to maintain the sludge concentration inside the reactor.

The study was conducted for 585, 200 and 250 days for SBR-1, -2 and -3, respectively. The end of each reactor's operational period was determined by its evolution. Due to the origin of the influent (Table 6.1), the SBRs had different OLRs even when working with the same VER. Figure 6.3 gives the OLRs obtained for the whole study for each reactor.

SBR-1, -2 and -3 presented OLR mean values of 0.9 ± 0.4, 0.5 ± 0.3 and 0.7 ± 0.3 Kg COD·$m^{-3}d^{-1}$, respectively. According to the literature, one of the main drawbacks of low-strength wastewater granulation is that OLR of around 1 Kg COD·$m^{-3}d^{-1}$ is still insufficient to obtain a biomass increase in time (de Kreuk and van Loosdrecht, 2006). The raw wastewater reactor (SBR-1) was the only one that reached a value close to this during the study. Variation in the influent due to the nature of real wastewater led to a higher OLR during operational periods in SBR-2 and SBR-3.

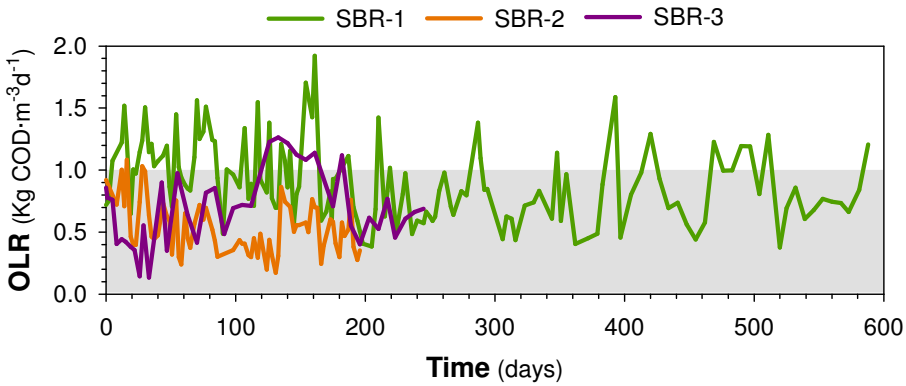

Figure 6.3. Organic loading rate (OLR) applied in SBR-1 (raw WW), SBR-2 (decanted WW) and SBR-3 (decanted + ethanol WW). Shadowed area indicates values under 1 Kg COD·$m^{-3}d^{-1}$.

A reduction in settling time for granulation purposes has been applied in other studies as well as in this one (see section 5.4.1.1). The method mainly affects biomass concentration and settling properties. Figure 6.4 shows the sludge volumetric index (SVI), mixed liquor suspended solids (MLSSs) and settling time in the treatment of different wastewater (WW) during this study.

As was expected, when the settling time was reduced, MLSSs decreased in all three reactors. Hence, at day 50, values of 2.0, 1.3 and 2.4 g MLSS·L^{-1} were

reached in SBR-1, -2 and -3, respectively. The biomass concentrations retained at the end of the settling time reduction were higher than those obtained with synthetic wastewater (Chapter 5). Settleability only improved in SBR-3 immediately after settling time was fixed at 2 minutes, with an SVI reduction from 300 to 50 mL·g^{-1} TSS.

However, when the three reactors were run at low settling times (2 minutes), different behaviors were observed. In the case of the raw WW reactor (SBR-1), biomass was continuously washed out from the system through effluent discharge until day 200. From that day on, SVI suddenly dropped and the sludge concentration started to recover. SBR-1 achieved stable conditions from day 400 to the end of the study with mean values of 4.5 ± 0.8 g MLSS·L^{-1} and 44 ± 8 mL·g^{-1}TSS.

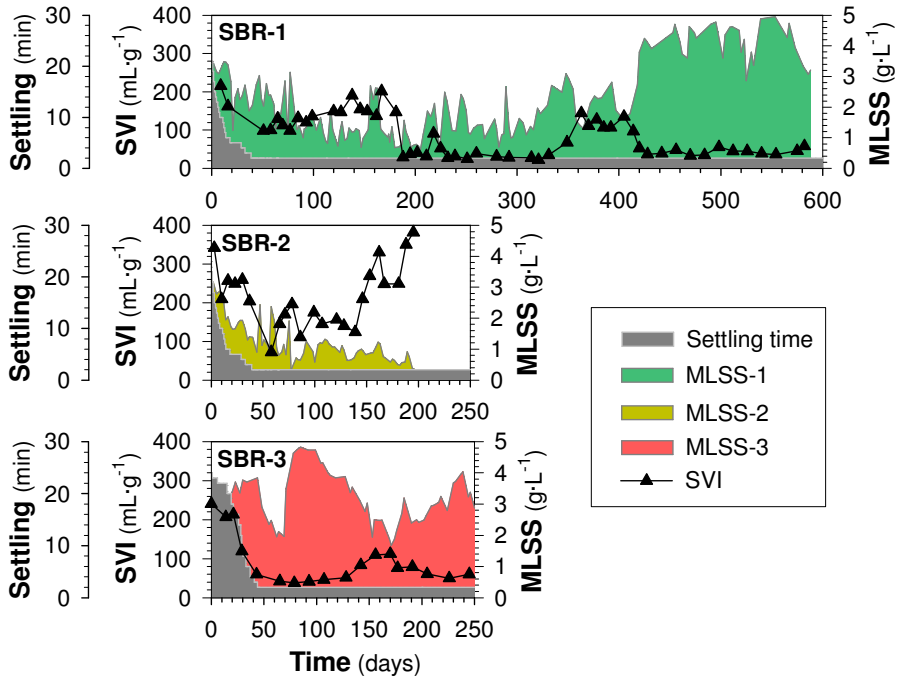

Figure 6.4. Sludge volumetric index (SVI), MLSS and settling time in SBR-1 (raw WW), SBR-2 (decanted WW) and SBR-3 (decanted + ethanol WW).

In the decanted WW reactor (SBR-2), the SVI improved slightly at the end of the settling time reduction (day 50). A bulking phenomenon from day 140 to the

end caused the SVI to rise to values of over 300 mL·g^{-1}TSS and, as a consequence, 0.4 g MLSS·L^{-1} was reached in the reactor, making it impossible to continue with the experiment. In SBR-3 (decanted WW with ethanol), settleability improved at the end of the settling time decrease and the SVI was maintained at under 100 mL·g^{-1} TSS until the end of the study. Biomass was maintained at higher concentrations than in SBR-1 and -2 at the end of the settling time decrease. Even though some washout of biomass was observed at certain times in the study, the minimum MLSS value was always over 2 g MLSS·L^{-1} in contrast with the other reactors.

Sludge settleability can indicate granule formation when a system achieves values lower than 100 mL· g^{-1} TSS (Beun *et al*., 2002a). However, higher values indicate a possible sludge washout through the effluent, which would severely affect its residence time and biomass concentration in the reactor. To evaluate these parameters, Figure 6.5 shows the evolution of SRT and MLSSs during the study period.

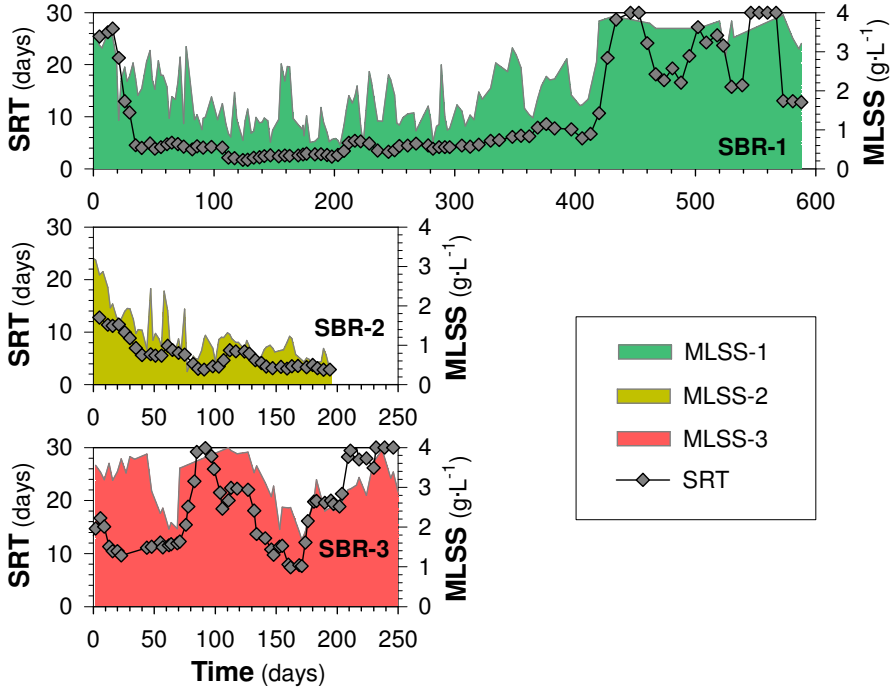

Figure 6.5. SRT and MLSS in SBR-1 (raw WW), SBR-2 (decanted WW) and SBR-3 (decanted + ethanol WW).

SRT is closely linked to the maintenance of biomass inside the reactor. When the settling time was reduced (from day 0 to 50), the SRT was reduced due to a wash out of sludge through the effluent. SRT values dropped to 4 days or lower in SBR-1 while the settling time was decreasing. This parameter was not recovered until MLSSs started to increase at day 200 and was not greater than 10 days until stable biomass concentration was achieved as of day 410. In the case of SBR-2, the progressive washout of biomass caused a similar profile for SRT values, which remained at between 3 and 6 days until the end of the study. In contrast, the higher amount of biomass in SBR-3 (3.2 ± 0.8 g MLSS·L^{-1}) allowed the SRT to increase to over 20 days. MLSS reduction around day 150 in this reactor caused the SRT to drop to 8 days. However, typical SRT values in this reactor were restored when biomass was recovered. Variations in the sludge age directly affect the growth of microorganisms, and because of this SRT is a major concern in the overall context of nutrient removal, as will be discussed later in this chapter.

According to the settleability and biomass profiles of the reactors (Figures 6.4 and 6.5) it seems that raw WW (SBR-1) and decanted WW dosed with ethanol (SBR-3) are the most suitable influents to promote granulation. These reactors also had the highest OLRs (Figure 6.3). To evaluate the performance of particle formation, size distribution during the study was analyzed and compared with stereomicroscope images. Figure 6.6 depicts the results obtained for SBR-1.

Size distribution analyzed by percentiles provides information of the granulation process. The 90^{th} percentile (P90) refers to the size that only 10% of the particles are over. The 50^{th} percentile (P50) is considered the median or the value which is found in 50% of the observations. Finally, the 10^{th} percentile (P10) is the minimum size reached by 90% of the sludge distribution. Low values such as 20 µm in the 10^{th} percentile indicate the presence of floccular sludge persisting in the reactor.

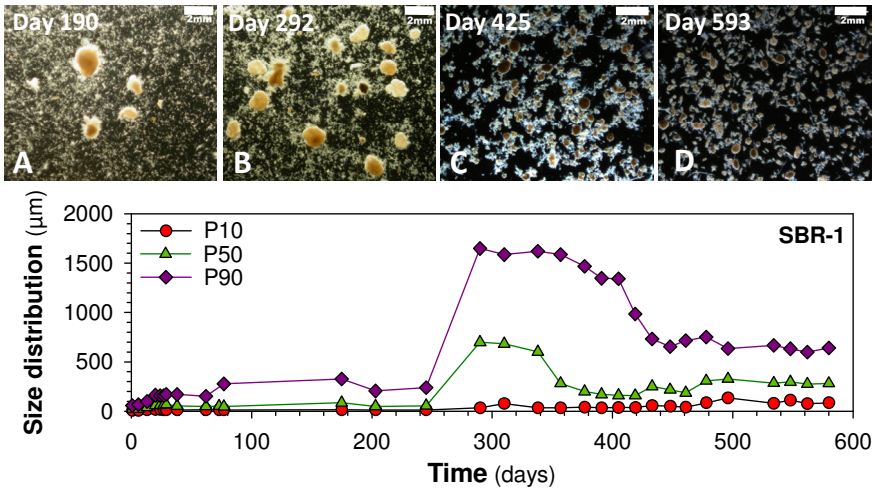

Figure 6.6. Stereomicroscope images (top) and 10th, 50th and 90th percentiles (P10, P50 and P90) (bottom) in SBR-1 (raw WW) during granulation study. Scale bars are at 2 mm.

Different behaviors regarding size distribution were observed during the SBR-1 operation. Percentile evaluation of the biomass (Figure 6.6, bottom) displayed four different distributions: from day 0 to 250 (A), from day 290 to 340 (B), from 380 to 405 (C), and from day 475 to the end of the study (D). Table 6.2 summarizes the mean values obtained during these periods.

Table 6.2. Summary of size distribution in SBR-1 treating raw WW. Units in µm.

	A Day 175-250	B Day 290-340	C Day 380-405	D Day 475-580
P10	14 ± 1	49 ± 25	38 ± 3	95 ± 23
P50	63 ± 19	660 ± 52	177 ± 20	295 ± 19
P90	257 ± 63	1617 ± 31	1384 ± 71	654 ± 52
MEAN	108 ± 22	729 ± 31	444 ± 45	348 ± 26

The tenth percentile (P10) tended to increase during long-term operation, although all values were less than 100 µm. This indicated the permanence of floccular sludge in the system. The mean size at which a particle is considered to be a granule (200 µm; de Kreuk *et al.*, 2007) was not reached until day 290 (Table 6.2B; Figure 6.6, bottom). Nevertheless the appearance of a few granules could be observed by the naked eye during the first part of the study (Figure 6.6A) coinciding with a slight increase in the 90th percentile. From day

250, an increase in P50 and P90 was observed, meaning granulation was taking place. Granules started to break up at day 380 as P50 was reduced from mean values of 660 to 177 µm. They continued to be disrupted, with P90 decreasing from 1384 to 654 µm at day 475. From that day on, smaller, stable granules were obtained with a mean size of 348 µm until the end of the operational period (Figure 6.6, Table 6.2) when a fully granular system was obtained with P10 presenting values of over 100 µm.

With regards to the performance of the reactor using decanted WW, Figure 6.7 shows the stereomicroscope and size distribution obtained during the study. SBR-2 did not present very great changes during the operational period. The maximum P90 reached was 200 µm while P50 only achieved 60 µm, indicating that floccular sludge was the major biomass in the reactor. Occasional granules were detected from stereomicroscope images, even though they had an amorphous morphology and they were surrounded by *vorticella*-like organisms (Figure 6.7B). This, together with filamentous growth, caused disturbances to the settling properties (Figure 6.4).

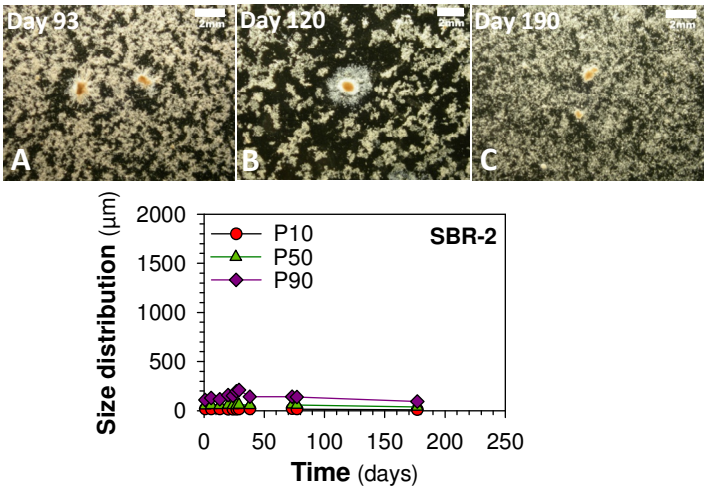

Figure 6.7. Stereomicroscope images (top) and 10th, 50th and 90th percentiles (P10, P50 and P90) (bottom) in SBR-2 (decanted WW) during granulation study. Scale bars are at 2 mm.

Granular formation in SBR-3 was also evaluated through stereomicroscope and size distribution analysis. The results obtained are depicted in Figure 6.8.

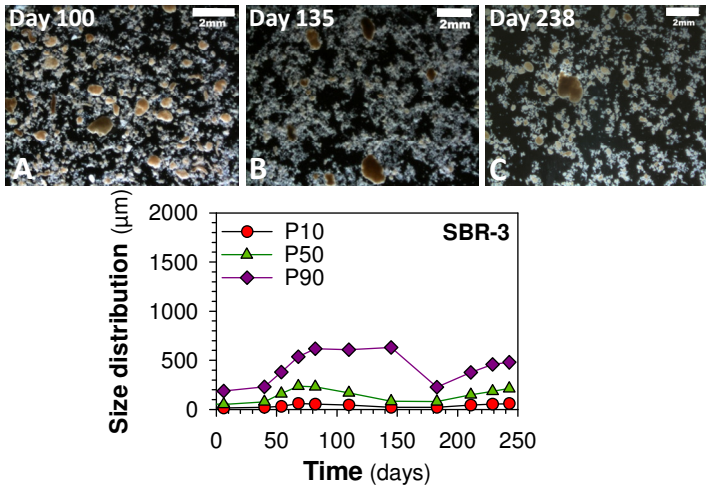

Figure 6.8. Stereomicroscope images (top) and 10th, 50th and 90th percentiles (P10, P50 and P90) (bottom) in SBR-3 (decanted WW with ethanol) during granulation study. Scale bars are at 2 mm.

SBR-3 started to form big particles after day 50 when the settling time was set at 2 minutes, with P50 and P90 reaching values of about 180 and 600 µm, respectively. This indicated the granulation of some of the biomass, but P10 of 45 µm showed that floccular sludge remained in the system (Figure 6.8A). The granules broke up from day 150 as the reduction in P90 denotes. However, all percentiles started to increase again at day 180 of the study when big particles were obtained (Figure 6.8C).

The granulation of floccular biomass when treating domestic wastewater was only achieved using raw WW and decanted WW supplied with ethanol, which both presented higher loading rates (Figure 6.3). Raw wastewater may enhance granulation performance because of particles that provide a starting point while the OLR is increasing. In the case of SBR-3, ethanol would provide ready biodegradable organic matter, thereby improving the growth of the bacteria. The key point here might be that the more conservative settling time reduction maintained the biomass concentration in the reactor. However, both reactors showed cyclical behavior in terms of MLSS concentration (Figure 6.4) and granulation (Figure 6.6 and 6.8). This was shown by an increase in MLSSs and percentiles when granules were developing and a decrease of the same parameters when they were breaking up.

6.4.2 Nutrient removal performance during granular development

Granular development is one of the big challenges when treating low-strength wastewater, and nutrient removal is the main purpose of the process focusing on domestic treatment. Neither biomass concentration (Figure 6.4) nor granular formation (Figure 6.7) were stable in SBR-2, so nutrient removal was not evaluated for this reactor.

Organic matter removals (data not shown) did not present any difficulty in the systems. COD removals efficiencies during the study were 88% and 89% for SBR-1 and -3, respectively, but there were different behaviors for nitrogen and phosphorus removal in these reactors.

6.4.2.1 Raw wastewater

SBR-1 treated raw wastewater, with a higher concentration of organic matter than the other influents (Table 6.1). Ammonium, total nitrogen, and phosphorus efficiencies with the OLR and settling times that were applied in SBR-1 are shown in Figure 6.9.

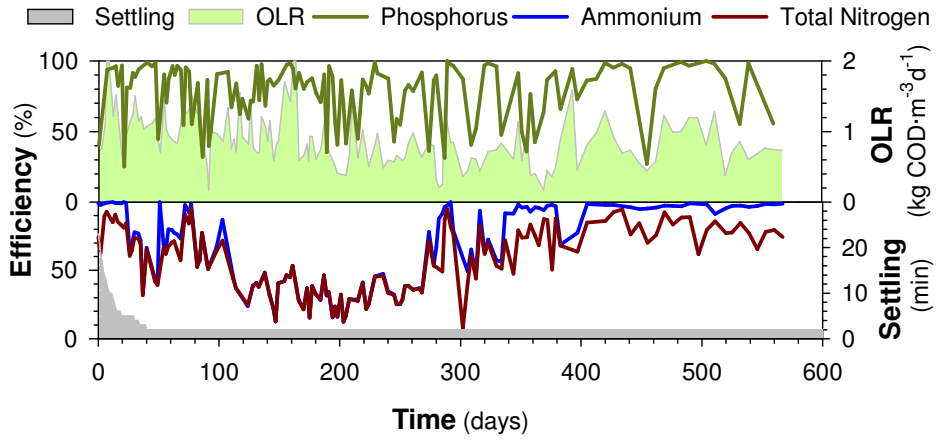

Figure 6.9. Phosphorus, ammonium, and total nitrogen efficiencies, organic loading rate (OLR) and settling time for SBR-1 (raw WW).

From the beginning, removal efficiencies were reduced and showed a lot of variation when a settling time decrease was applied. The study of the raw wastewater was divided into four different periods according to organic loads and removal efficiencies (Table 6.3).

Table 6.3. Influent organic matter and mean removal efficiencies for SBR-1.

Period Days	A 0-110	B 110-270	C 270-370	D 370-end	Units
COD_T	628 ± 170	540 ± 166	459 ± 176	552 ± 188	mg $COD \cdot L^{-1}$
COD_S/COD_T	38	43	34	51	**%**
Ammonium	78 ± 19	33 ± 10	78 ± 22	93 ± 10	**%**
Total nitrogen	69 ± 15	32 ± 10	65 ± 19	71 ± 25	**%**
Phosphorus	76 ± 25	72± 24	61 ± 31	79 ± 27	**%**

Nitrification efficiency started to decrease from the beginning of the study and achieved minimum values of 33% at day 110 (Figure 6.9). The loss of ammonium oxidation was due to a reduction in the SRT from 25 to 3 days (1.5 days of aerobic SRT, SRT_{AER}; see section 3.4.2.1) when efficiency was at its minimum (Figure 6.5, top). However, nitrification clearly recovered from day 170 although there was a low SRT of 5 days. This was related to the development of granules, which retain the biomass in the reactor for a longer time than floccular sludge. Thus, calculated SRT for granular systems is always lower than the real SRT because only slow settling particles are withdrawn from the reactor. Finally, maximum nitrification efficiency (Table 6.3) was obtained from day 370 when the biomass was mainly formed by granules (Figure 6.6).

After the recovery of ammonium removal, a bigger difference between nitrification efficiency (blue line, Figure 6.9) and total nitrogen efficiency (red line, Figure 6.9) was observed. This indicates incomplete denitrification or incomplete hydrolysis of the organic nitrogen (TN = Norg + NH_4^+ + NO_x^-) and there were huge fluctuations in this difference during the study. Hydrolysis of organic nitrogen usually happens under anaerobic conditions, while these huge variations seemed to be related to the OLR (Figure 6.9), which would be evidence of substrate limitation in the BNR process. This was suggested by a wider difference between nitrification and total nitrogen efficiency when OLR decreased. The raw wastewater only contained 43% of soluble organic matter

(COD_S/COD_T, Table 6.3), while the rest was in the form of particulate COD. The denitrification performance was evaluated through nitrogen balances and is shown in Figure 6.10. The theoretical NO_x^- concentration, as the sum of nitrite and nitrate, was calculated as the complete oxidation of the ammonium added in the second feed event, assuming complete denitrification in the previous anoxic phase. If the difference between experimental and theoretical values is positive, NO_x^- is accumulated in the reactor. Conversely, when the values are negative, simultaneous nitrification denitrification (SND) is performed under aerobic conditions (see section 3.4.4).

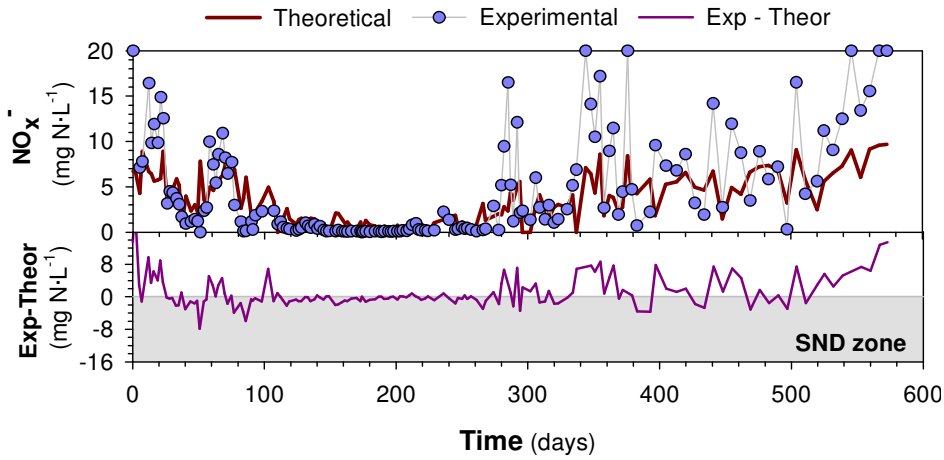

Figure 6.10. Theoretical and experimental nitrate and nitrite (NO_x^-) effluent concentrations (top) and nitrate and nitrite removed by SND (experimental – theoretical) (bottom) in SBR-1 (raw WW).

Nitrate accumulation and some periods of SND were observed during the granulation period (from day 0 to 100). From day 100 to 240 nitrification did not occur (theoretical and experimental values were close to 0 mg $N\text{-}NO_x^-\cdot L^{-1}$) (Figure 6.10, top). After granulation was achieved (from day 300 to 500) (Figure 6.6, bottom), ammonium removal was recovered and there was an SND tendency in the system. However, poor denitrification under anoxic conditions and, consequently, nitrate accumulation were observed in most of the cases because of a lack of organic matter (OLR, Figure 6.9, top). Furthermore, SND was not enhanced when small and homogeneous granules were obtained from day 500 to the end of the study (348 µm, Table 6.2). Because SND did not occur, NO_x^- accumulation in the effluent was not reduced.

There were big fluctuations in phosphorus removal efficiencies, which decreased when the OLR was also reduced (Figure 6.9, top). It has to be taken into account that only a fraction of COD from the influent was readily biodegradable (Table 6.3). When ammonium removal efficiency was very low (Table 6.3, column B), the mean phosphorus efficiency reached values of 72%, higher than when nitrification was recovered (61%, Table 6.3, column C). This could have been due to organic matter competition between the denitrification and phosphorus removal processes. From day 370 to the end of the study, a higher OLR was observed and phosphorus removal increased to 79% (Table 6.3, column D).

The low biodegradable COD from raw wastewater affected both denitrification and phosphorus removal (Table 6.3). However, it should be noted that hydrolysis of organic particles can take place under anaerobic conditions, and in the process increase soluble COD and improve phosphate release. In order to evaluate the effect of the available COD on EBPR, tests with an anaerobic phase of normal length (170 minutes, the one applied in the SBR) and a longer phase of 210 minutes were carried out. Figure 6.11 shows the results obtained at day 400 of the operational period when granules presented a mean size of 444 µm and both phosphorus and total nitrogen reached efficiency values of around 80%.

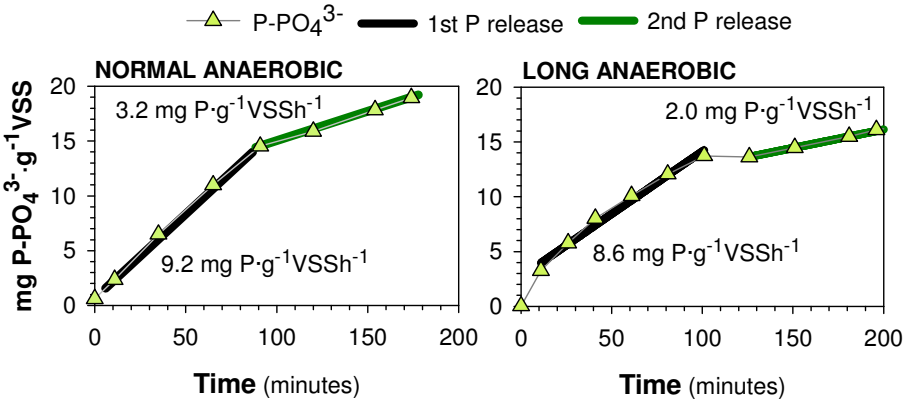

Figure 6.11. Phosphate ($P\text{-}PO_4^{3-}$) concentrations under anaerobic conditions for phases of a normal length (left) and longer (right) at day 400 of the study. Fit curves stand for phosphate releases.

According to the tests with normal and longer anaerobic phases, phosphate was released at around 9 mg $P \cdot g^{-1}VSSh^{-1}$ after domestic wastewater was added. The first P release ended in both experiments at 100 minutes of anaerobic conditions. After this, a second P release with 3-4 times less velocity (3.2 and 2.0 mg $P \cdot g^{-1}VSSh^{-1}$ in normal and longer anaerobic phases, respectively) was observed. According to the literature, there should have been a second P release of about 0.4-0.6 mg $P \cdot g^{-1}VSSh^{-1}$ due to the maintenance energy required by phosphorus accumulating organisms (PAOs) (Puig *et al*., 2008). However, the values presented in these tests were far too big to be due to maintenance energy, and the second release under the anaerobic phase can be attributed to a second uptake of hydrolyzed organic matter coming from particles present in the influent. Making the anaerobic phase longer after the first release finish (first 100 minutes) would make biodegradable COD available for EBPR purposes, despite the soluble COD contained in the wastewater having already been taken up. The increase of 40 minutes of anaerobic phase did not suppose a huge enhancement on carbon uptake because of the lower values of the second P release. Therefore, it was decided not to make the anaerobic phase longer at the expense of the aerobic or anoxic phases, which could have affected the other BNR processes.

Raw wastewater is composed of a variety of substrates and, as previously noted, some of them may be available after hydrolysis. This may also influence internal polymer generation within PAOs. An internal polymer analysis from the poly-β-hydroxialkanoates (PHA) of raw wastewater is presented in Table 6.4 and a comparison is made with decanted wastewater treated in a floccular reactor under the same conditions.

Table 6.4. Composition of PHA accumulated as PHB, PHV and PH2MV in raw and decanted wastewater.

	PHB %	PHV %	PH2MV %
Raw WW	77.8	20.6	1.6
Decanted WW	89.7	10.3	0

As was expected from the real wastewater, in which has acetate is the main product of fermentation (Oehmen *et al.,* 2007), PHA was mainly composed of

PHB. Nevertheless, in the raw wastewater there was more PHV and PH2MV than in the decanted WW. As the main difference between raw and decanted wastewater was the particulate fraction, this increase in PHV and PH2MV was attributed to the products of hydrolysis. Particles in wastewater produce a PHA composition different from that of settled wastewater and it could affect the EBPR performance because of different metabolic pathways used for PHA oxidation under aerobic conditions.

Finally, to show the performance of BNR within the reactor, Figure 6.12 presents nitrogen and phosphorus profiles from a cycle study at day 413 of the study when stable granules and stable nutrient removal were achieved.

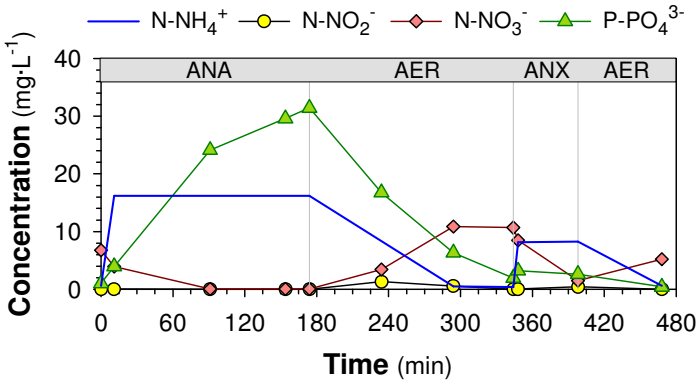

Figure 6.12. Cycle study of ammonium ($N\text{-}NH_4^+$), nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$) and phosphate ($P\text{-}PO_4^{3-}$) profiles for SBR-1 (raw WW) at day 413 of the study.

Organic matter from the influent was taken up by PAOs, releasing phosphate under anaerobic conditions. In the subsequent aerobic phase, phosphate was taken up while nitrification occurred and nitrate was produced. The difference between ammonium oxidized and nitrite produced could have been due to the combination of denitrification by PAOs (DPAOs) and ammonia assimilation by heterotrophic growth (Tchobanoglous *et al.*, 2003). At the end of the cycle, ammonium and phosphorus were completely removed from the system, while nitrite lower than the legal limit (15 mg $N{\cdot}L^{-1}$) were discharged through the effluent.

6.4.2.2 Decanted wastewater dosed with ethanol

Ethanol was used as an external carbon source to supplement decanted wastewater in SBR-3. Similar OLR values as in raw wastewater were applied. The main difference between SBR-3 and SBR-1 was the quantity of suspended solids in the influent, which accounted for higher total organic matter (COD_T, Table 6.1) than in decanted WW. Nitrogen and phosphorus efficiencies and the OLR and settling time applied in SBR-3 are shown in Figure 6.13.

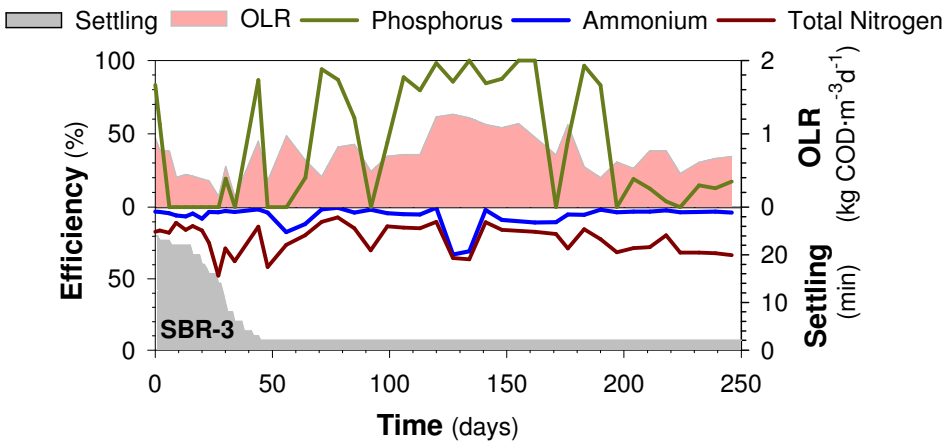

Figure 6.13. Phosphorus, ammonium and total nitrogen efficiencies, organic loading rate (OLR) and settling time in SBR-3(decanted + ethanol WW).

The increase in soluble organic matter due to the ethanol improved the granulation process and let to a lower washout of the biomass and adequate SRT in the reactor (Figure 6.5). This was reflected in the nitrogen removal efficiencies, since ammonium removal (blue line, Figure 6.13) was maintained at over 89% throughout nearly the whole of the study regardless of the conditions applied for granulation purposes. Total nitrogen efficiency (red line, Figure 6.13) was associated with the OLR applied in the reactor and three periods could be differentiated. Table 6.5 summarizes the organic matter in the influent and nitrogen and phosphorus removal in SBR-3.

Table 6.5. Influent organic matter and mean removal efficiencies in SBR-3.

Period Days	A 0-100	B 100-180	C 180-end	Units
COD_T	383 ± 169	673 ± 218	448 ± 144	mg $COD \cdot L^{-1}$
COD_S/COD_T	58	71	70	**%**
Ammonium	96 ± 4	89 ± 11	97 ± 1	**%**
Total nitrogen	79 ± 11	80 ± 10	73 ± 6	**%**
Phosphorus	25 ± 35	87 ± 16	26 ± 34	**%**

Total nitrogen efficiency was 79% during the first period. The difference between this value and ammonium removal efficiency is due to poor denitrification. According to the literature, the COD/N ratio for denitrification, taking into account biomass growth, will normally be around 4 Kg $COD \cdot Kg^{-1}$ N. However, optimal total nitrogen efficiency is achieved when the ratio is kept in the range of 5-7.1 Kg $COD \cdot Kg^{-1}$ N (Xiaolian *et al.*, 2006). The experimental COD/N ratio from SBR-3 was 9 ± 4 Kg $COD \cdot Kg^{-1}$ N, so denitrification should occur in the reactor. However, when the biodegradable fraction (COD_S/COD_T) (Table 6.5) is taken into account, denitrification could be affected in some periods of the operational period.

The increase in available organic matter from day 100 (Table 6.5) enhanced denitrification performance and reduced the difference between total nitrogen and ammonium efficiencies (Figure 6.13). In order to properly evaluate the denitrification performance of the reactor, theoretical and experimental NO_x^- in the effluent were calculated (see section 3.4.4). Figure 6.14 presents the results obtained.

Nitrification occurred throughout the study as shown by the fact that theoretical values from the last aerobic phase of the cycle (Figure 6.1) differed from 0 mg $N \cdot L^{-1}$. In terms of organic matter availability (Table 6.5A), accumulation of nitrate was evidenced by positive values in the difference between experimental and theoretical NO_x^-. However, between days 50 and 180 no nitrogen was accumulated. The lack of organic matter during the final days of the study (Figure 6.5C) led to a nitrogen accumulation in the effluent (Figure 6.14). At the end, SND was not considered in this reactor as small

negative differences between experimental and theoretical values could have been due to ammonium assimilation.

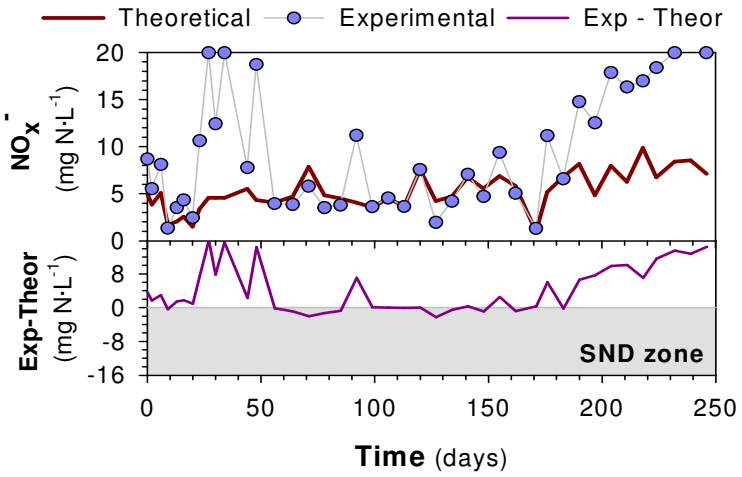

Figure 6.14. Theoretical and experimental nitrate and nitrite (NO_x^-) effluent concentrations (top) and nitrate and nitrite removed by SND (experimental – theoretical) (bottom) in SBR-3 (decanted + ethanol WW).

There was wide variation in phosphorus throughout the entire study, as the big standard deviation values in Table 6.5 show. EBPR was only observed when denitrification was improved and higher OLRs were applied (Figure 6.13). From day 100 to 180 (Table 6.5B) a higher amount of organic matter enhanced phosphorus removal and achieved efficiencies of 87%. However, even though the percentage of COD_s/COD_T was maintained from day 180, organic matter was reduced and EBPR, as well as denitrification, were affected, lowering again the removal efficiency to 26%. This shows that availability of organic matter was the limiting factor for nutrient removal throughout the study.

6.4.2.3 Effect of temperature in a BNR granular system

Temperature is one of the main parameters affecting the bacterial growth that is responsible for nutrient removal and granule formation. In this study the temperature of the reactor behaved in line with the ambient temperature of the laboratory, and therefore a dynamic profile based on seasonal changes was obtained during the operation of the SBR. The minimum SRT that allow the

development of autotrophic microorganisms (the slowest growers in BNR systems) was calculated as a function of temperature (see section 3.4.2). Figure 6.15 shows the efficiency of nitrogen removal together with the ratio of real and minimum aerobic SRT, as well as reactor temperature and biomass mean size during the entire study in SBR-1.

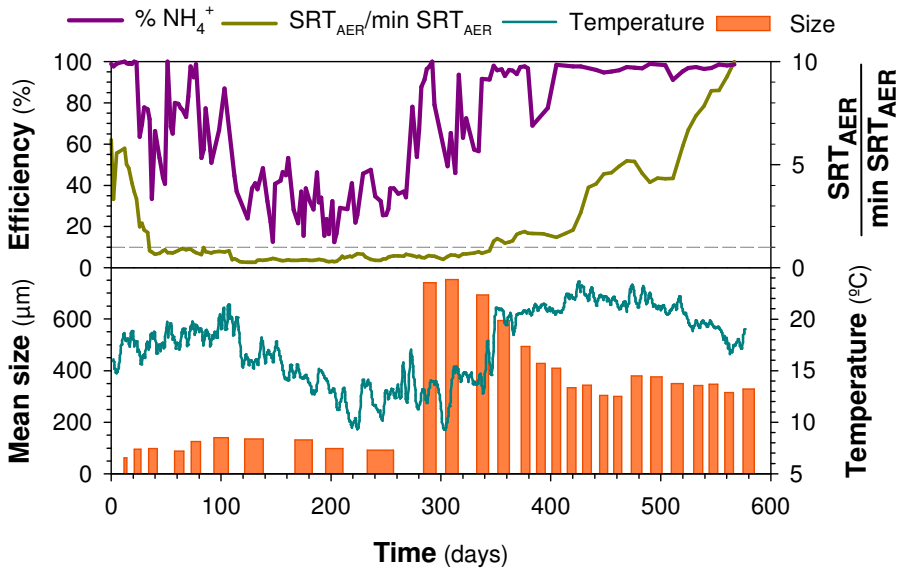

Figure 6.15. Ammonium (NH_4^+) removal efficiency and real and minimum aerobic SRT ratio (SRT_{AER}/min SRT_{AER}) (top) and mean size in relation to the temperature (bottom) in SBR-1.

Nitrification, and as a consequence total nitrogen removal, is possible when the real and minimum SRT ratio is equal to or over 1. For this reason, total nitrogen started to decrease when the settling time was reduced (from day 0 to 50) and SRT_{AER}/minSRT_{AER} dropped from 6.2 to 0.8 (Figure 6.15, top). Despite the SRT ratio being maintained at a low value of about 0.8, ammonium removal efficiency of 74% was obtained from day 50 to 100. After this, ammonium efficiency diminished greatly and reactor temperature decreased until day 290 (Figure 6.15), when granules with a mean size of 700 µm suddenly developed and ammonium efficiency recovered. At this stage, floccular and granular biomass coexisted in the reactor (Figure 6.6). The settleability of the two biomasses was different and floccular biomass was washed out from the reactor. As granular biomass was retained in the system, the sludge inside the

reactor presented a higher SRT, allowing nitrification to occur even though the calculated SRT was still insufficient for ammonium removal (Figure 6.15). Finally, when optimal values of SRT and temperature where achieved at day 340 (an SRT ratio higher than 1 and temperature around 20ºC), nitrification was enhanced despite the mean size of the granules being under 400µm.

The minimum temperature was around 12ºC when the bigger granules (700 µm) developed in the system (Figure 6.15). This could have been due to a greater stress in the reactor and granular development, so colonization and further aggregation provided a safe growth environment for microorganisms, one in which they are protected from the adverse effects of environmental conditions (Dulekgurgen *et al*., 2008b). However, granules also remained in the system after they had broken up, even though higher temperatures were observed. Thus, no conclusions can be drawn from the effect of temperature on granulation and further research is required.

The same evaluations of temperature, SRT, and mean size were done for SBR-3 and are shown in Figure 6.16.

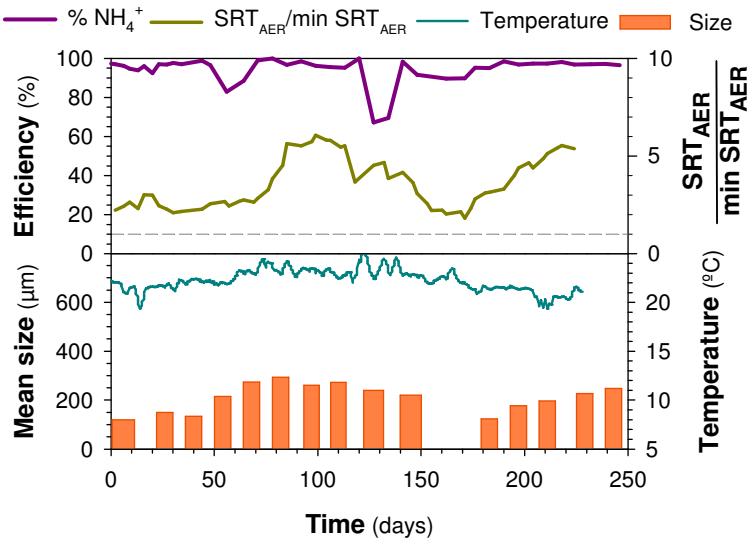

Figure 6.16. Ammonium (NH_4^+) removal efficiency and real and minimum aerobic SRT ratio (SRT_{AER}/min SRT_{AER}) (top) and mean size in relation to temperature (bottom) in the SBR-3.

In this reactor the temperature was maintained at over 20ºC throughout the operational period and the $SRT_{AER}/minSRT_{AER}$ ratio was over 2, meaning that nitrification was not affected by either temperature or SRT limits. Granule size achieved a maximum mean size of 290 µm and no correlation with temperature was observed.

6.4.3 Microbial distribution in domestic granules

Fluorescence in situ hybridization (FISH) is used on sludge samples to determine the presence of different species communities and quantify them within the biomass. Coupling this molecular technique with cryosection method made it possible to get an overview of the nitrogen and phosphorus microorganisms distribution into the particles. Figure 6.17 depicts the results obtained from FISH micrographs at day 320 of the study when analyzing PAOs, glycogen accumulating organisms (GAOs) and ammonium oxidizing bacteria (AOB) bacteria in relation to the total bacteria from SBR-1.

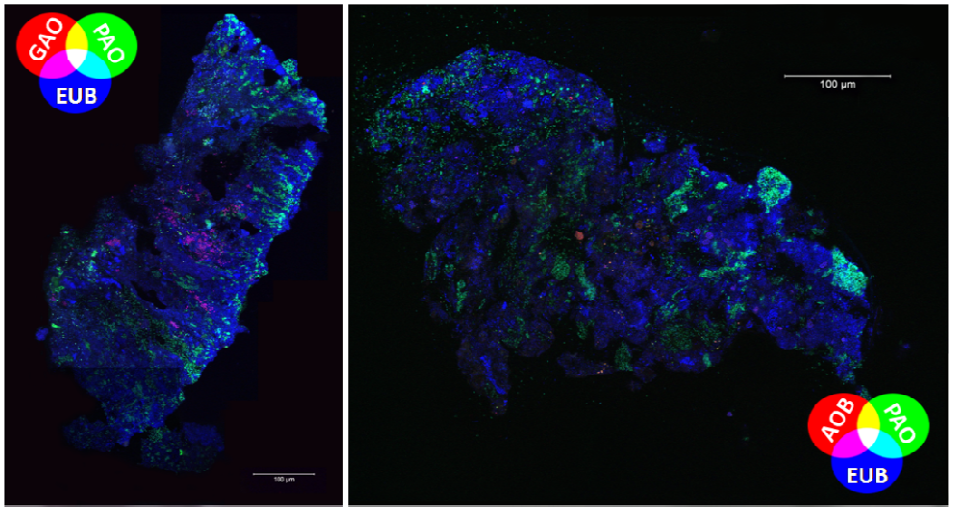

Figure 6.17. FISH micrographs of granules at day 320 of the study stained with CY-3 (red) for GAO (left) and AOB (right), FITC (green) for PAO and CY-5 (blue) for all bacteria. Scale bar of 100 µm.

Granules were obtained from SBR-1 in the middle of the long-term study. As can be seen from Figure 6.17, there were irregularities in the internal parts of the structures. The black zones represent empty holes or dead biomass inside the granules.

PAOs and GAOs can be either aerobic or anoxic, and for this reason it was expected to find these microorganisms all over the particles (Figure 6.17, left). However, it seemed that PAOs, which accounted for 40% of the total bacteria of the granule, were more active (with a higher signal intensity) on the outer part even though they were spread out within the entire particle. In contrast, GAOs, which accounted for 15% of the total bacteria population of the granule, were mainly found in the inner part of the aggregate.

AOB are completely aerobic, and therefore they should be found in the outer layers of the granules where oxygen is readily available. In the case of domestic granules, about 10% of the AOB were found on the outer parts of the aggregates, but some colonies were detected in the core as well (Figure 6.17, right). This could be explained by the fact that domestic granules appear to be less compact than particles obtained with high organic loading rates and synthetic wastewater. The lack of density inside the granules allowed some channels to be formed which nutrients as well as oxygen could go through. The smaller size of granules when treating urban wastewater (Figure 6.21) reduced the problem of oxygen diffusion into the core of the biomass aggregates.

One key fact to emerge from these microbiological analyses was that PAOs were situated all around and inside the granules, and achieved a higher population than the usual percentage of 5-15% in floccular EBPR systems (Lopez-Vazquez *et al.*, 2008). Therefore, we can postulate that PAOs are one of the key factors when it comes to obtaining granular sludge. However more research needs to be carried out in this direction.

6.5 Discusion

6.5.1 Parameters affecting granulation when treating real domestic wastewater

Domestic wastewater is a stream containing different compounds of organic matter, of nitrogen and of phosphorus that can be present in particulate or in soluble fractions. Domestic wastewater treatment is characterized by a primary treatment were the particulate fraction is separated so that the soluble part can be easily treated using activated sludge systems. However, for granular systems, particles present in the influent could be one of the factors behind granulation. First, particles may be the initial support which biomass attaches itself to and grows, making the initial granules easier to obtain. Second, in terms of organic matter, particles increase the organic loading rate of the system as compared with decanted influents, which has been found to be the bottleneck in granular development.

According to the results given in this chapter, granules appeared when treating either raw wastewater or decanted wastewater dosed with ethanol, but not when using decanted wastewater as the sole influent. However, there were cyclical fluctuations in both systems in terms of granular stability, with mean size and MLSSs being reduced when granules broke up. Furthermore, the start-up with raw wastewater was longer than with decanted wastewater dosed with ethanol. This might be due to a lower availability of ready biodegradable organic matter from raw wastewater or a more conservative settling time decrease in decanted wastewater with ethanol. This means that not only OLR is important for granulation; the quantity of organic matter available is as well.

6.5.2 Nutrient removal effects in domestic granules

The stability of nitrogen and phosphorus removal has been affected when granulation takes place, whichever the influent treated, until the system achieves a steady state. However, when dealing with domestic wastewater, the low easily biodegradable organic matter content makes a BNR system more

unstable when biomass starts to be washed out of the system because of a reduction in settling time. Maintaining the settling time at 2 minutes while flocs are the major biomass in the reactor causes instability in ammonium oxidation there is a low SRT. In contrast, granular development favors nitrification even if the calculated SRT presents low values. This was explained by the fact that the real SRT is higher than the one calculated, as biomass is retained for a longer time entrapped with the granules.

In order to complete total nitrogen removal, denitrification needs to be promoted after nitrification is recovered. SND is usually enhanced by diffusion through the granules when organic matter is available under aerobic conditions or denitrification is performed by DPAOs. In cases similar to this study in which low-strength wastewater is treated and organic matter is a limiting parameter, virtually no SND can occur, and nitrate is accumulated in the effluent.

The bottleneck in phosphorus removal performance has always been the availability of a carbon source during anaerobic periods. In cases were denitrification is not completely achieved, carbon uptake is greatly reduced and, as a consequence, phosphate is taken up from the media. However, when optimal conditions are applied to increase the PAO population, these organisms grow all over the particles and form big colonies that enhance the compactness of the sludge. Accordingly, the application of higher loading rates would enhance granulation not only because of an increase in heterotrophic growth, but also because PAOs would be enriched forming big colonies that would improve the aggregation of the biomass. Furthermore, hydrolysis of particles from raw wastewater would release different compounds that would increase the availability of organic matter and produce a major diversity in storage polymers for EBPR.

6.6 Conclusions

Raw wastewater has been proved to be the most suitable for granular formation because higher loads are obtained with this type of influent than with decanted wastewater. However, a long start-up is required with low-strength influents, even when treating raw wastewater. In this study, decanted

wastewater dosed with ethanol needed a shorter start-up for granulation purposes. Two hypotheses have been considered to explain this: i) a higher concentration of ready biodegradable organic matter; and ii) a more conservative settling time decrease, thereby avoiding sludge washout from the system.

Nutrient removal is not directly affected by the granulation process, but as a consequence of biomass washout during the start-up. However, the development of big granules can increase the real SRT as biomass is retained longer in the reactor, thereby enhancing nitrification recovery. The bottleneck in granulation for nitrogen and phosphorus removal purposes has been proven to be the availability of organic matter for both particle enhancement and nutrient removal. This organic matter can come either from the hydrolysis of organic particles or from the addition of an external carbon source.

DISCUSSION

Chapter 7. General discussion

This chapter outlines the general discussion of this thesis and summarizes its contribution to the study of biological nutrient removal and granulation.

The research line in which this thesis has been focused is biological nutrient removal from domestic wastewater. Although a lot of work has been done to acquire basic knowledge in this field in recent decades, operational problems are still found in wastewater treatment plants. Continued population growth and increasing demands for water have driven the search for more compact and stable systems. As a result, granular reactors have been proposed as a suitable technology for wastewater treatment. In the sections that follow, the factors affecting nutrient removal and granulation in domestic wastewater treatment are discussed.

7.1 Granulation

According to the literature, granules have been obtained by applying high organic loading rates (OLRs) and short settling times (Liu and Tay, 2004; de Kreuk *et al*., 2007; Adav *et al*., 2008a). Against this background, the following factors were investigated in this thesis: i) progressive settling time reduction to select the fastest particles from the biomass; ii) an increase in the volume exchange ratio (VER) to increase the OLR; and iii) the use of raw wastewater in place of decanted wastewater to increase the OLR.

Settling time reduction has been found to be effective for granulation purposes. An exponential decrease in the settling phase length allows the system to acclimate itself as slower settling particles are progressively washed out from the reactor. However, when working with low-strength wastewater, the low level of organic matter does not allow sufficient growth of microorganisms to stabilize the biomass concentration. For this reason, a more conservative settling time reduction, for example one in accordance with experimental sludge observations, maintained the solids concentration in the reactor while enhancing granulation.

The main difference when applying a different VER in an SBR, which increases the OLR proportionally, is the recovery of MLSS concentration and the size and shape of the granules obtained. In this study, at the lowest VER of 40%, granules reached their biggest mean diameter and highest biomass

concentration. Instead of enhancing granulation when the VER was increased to 50%, as might have been expected because of the increase in the OLR, biomass concentration slowed down due to an increase in the amount of treated water discharged and, consequently, a higher washout of particles. This was aggravated when a 60% VER was used, when it was even more difficult for granules to develop. When the OLR is increased with a higher VER and biomass concentration is not improved, more organic matter is available, thereby allowing a filamentous outgrowth. When filaments dominate the reactor, they entrap all particles from the biomass, which has a negative effect on the settling properties of the system.

Particles present in domestic wastewater are considered one of the key factors in granulation because they may be the initial support to which biomass attaches itself, thus making it easier for the initial granules to develop. However, granules can appear when either raw wastewater or decanted wastewater dosed with ethanol is being treated, but not when decanted wastewater as the sole influent is used. Thus, the OLR provided by the particulate fraction of the influent or the dosed external carbon source may have been the parameter enhancing granulation. Furthermore, the start-up with raw wastewater was longer than that with decanted wastewater dosed with ethanol. This might have been due even to a lower availability of ready biodegradable organic matter from raw wastewater or a more conservative settling time decrease in decanted wastewater with ethanol. This shows that not only the OLR is important for granulation, the quantity of available organic matter is as well.

Granular performance in terms of mixed liquor suspended solids (MLSSs) and size was dynamic when low-strength wastewater was being treated for both synthetic and real influents. Cyclical profiles were observed within biomass concentration. Immediately a maximum amount of solids inside the reactor was reached, granules were disrupted, and the smallest particles were washed out through the effluent causing a decrease in MLSSs. This cyclical behavior was proportionally related to the sludge residence time (SRT), which also affected nutrient removal performance. Granulation in low-strength wastewater is possible, but further research needs to be carried out to deal with the

bottleneck in the process, which is the instability of the system until a fully granular reactor is obtained.

7.2 Nitrogen removal

Nitrogen is removed biologically first by ammonium oxidation first and then by denitrification. Nitrification is the most sensitive process after a loading increase because of the slow growth of nitrifying bacteria. It is the first process to be recovered as it is severely affected by a low SRT, which happens when insufficient biomass has grown to compensate for the loading increase. When ammonium oxidation is enhanced, nitrate in the reactor increase. Denitrification efficiency is the following target, as nitrate is the major compound interacting with phosphorus removal.

When trying to improve nitrification, an aerobic SRT (SRT_{AER}) of over four days (at 20ºC) is essential. This can be achieved by increasing the aerobic phase at the expense of part of the anaerobic phase. However, transient response periods when conditions are being changed (from anoxic to aerobic) have to be considered as they can limit the growth of the nitrifiers. Reducing the step-feed events by joining together the aerobic phases, thereby obtaining a longer aerobic period, will achieve maximum bacterial activity and improve ammonium removal from the media. Furthermore, diffusion phenomena around the activated sludge flocs (and even more so in the presence of granular sludge) might be a determining factor in the transient phenomenon. Even with a similar SRT, longer aerobic periods and reduced alternating conditions enhance ammonium oxidation after a load increase.

One of the problems of the granulation strategy is that maintaining the settling time at 2 minutes while flocs are the major biomass in the reactor causes instability in ammonium oxidation because of the low SRT. In contrast, granular development favors nitrification even if the calculated SRT is low. This can be explained by the fact that a real SRT is higher than a calculated one, as biomass is retained for a longer time entrapped with the granules.

When recovering nitrification after the SRT has risen sufficiently, ammonium oxidizing bacteria (AOB) can grow as floccular sludge or around particles surfaces. As a result of a lack of substrate (nitrite), nitrite oxidizing bacteria (NOB) takes longer to develop and nitrite can be released in the effluent. The time to achieve nitratation (oxidation of nitrite to nitrate) is usually short in conventional floccular sludge and only some nitrite is accumulated in the effluent. However, the presence of granules made that period longer and nitrite was observed in the effluent during some days. Therefore, granular sludge would delay the nitratation process.

Later, in order to complete total nitrogen removal, denitrification needs to be promoted after nitrification is recovered. The water distribution in a step-feed strategy could prevent nitrogen accumulation in the effluent. Applying a higher volume of water in the first feed event reduces nitrate in the effluent if the subsequent feed event provided enough organic matter for denitrification.

7.3 Phosphorus removal

The presence of nitrate and/or nitrite destabilizes enhanced biological phosphorus removal (EBPR) performance due to organic matter competition. When nitrite is the intermediate of nitrification-denitrification in the system, inhibition by free nitrous acid (FNA) severely affect phosphorus removal, reducing the phosphate uptake rate (PUR) in the short term and the phosphorus accumulating organisms (PAO) population in the long term. Hence, changing wastewater distribution in the feeding events by increasing the first feed at the expense of the second is one of the possible solutions tested in this chapter. A higher volume distribution in the first part of the cycle makes organic matter available for EBPR while less nitrate concentration is obtained in the effluent. With regards to phosphorus removal, anaerobic length has to be adjusted after increasing the volume fed in order to allow all the organic matter to be taken up and stabilize the phosphate release.

The bottleneck in phosphorus removal performance has always been the availability of a carbon source during anaerobic periods. In cases where

denitrification is not completely achieved along the cycle and nitrates remain in the anaerobic phase, carbon source is consumed for nitrate removal and it is greatly reduced for PAOs. As a consequence, poly-β-hydroxyalkanoates (PHAs) are not formed and phosphate is not taken up from the media in the subsequent aerobic phase. However, when optimal conditions are applied to increase the PAO population, these organisms grow all over the particles, forming big colonies that enhance the compactness of the sludge. Accordingly, the application of higher loading rates enhances granulation not only because of an increase in heterotrophic growth, but also because PAOs are able to be enriched and the aggregation of the biomass improved. Furthermore, the hydrolysis of particles from raw wastewater releases different compounds that increase the availability of organic matter and produce major diversity in storage polymers for EBPR.

7.4 Simultaneous processes

Granules allow different conditions (anaerobic, anoxic and aerobic) in the same tank due to oxygen diffusion limitation. Because of this, simultaneous processes such as simultaneous nitrification denitrification (SND) or even simultaneous nitrification, denitrification, and phosphorus removal (SNDPR) can be used. SND should be enhanced by diffusion through the granules when organic matter is available under aerobic conditions or denitrification is performed by denitrifying PAOs (DPAOs).

The application of granular sludge could therefore be helpful in SNDPR. DPAOs use both nitrite and nitrate for phosphorus removal in granular sludge, with similar DPAO activity. However, in this study a higher phosphorus uptake – nitrogen removal (P_{UP}/N_{REM}) ratio was found when using nitrate rather than nitrite as the electron acceptor. This could be because of the higher electron demand from nitrate (5 electrons) than nitrite (3 electrons) during denitrification. Taking into account the requirement for organic matter for denitrification (Tchobanoglous *et al.*, 2003), nitrite was found to be more effective for phosphate removal when organic matter limitations affected the system from which it can be concluded that the use of either nitrate or nitrite should be evaluated in terms of the system's requirements.

CONCLUSIONS

Chapter 8. General conclusions and future perspectives

This chapter gives an overview of the main contributions of this PhD thesis to the study of nutrient removal interactions when moving from floccular to a granular sludge system.

The thesis deals with the improvement of biological nutrient removal (BNR) in a sequencing batch reactor (SBR) treating domestic wastewater. In particular, the work presented evolves from the application of optimal operating conditions for BNR recovery of a floccular sludge system to the attainment of a granular reactor suitable for low-strength wastewater treatment.

8.1 Nitrogen and phosphorus removal restoration in a floccular sludge SBR

Nitrogen and phosphorus removal destabilization due to an increase in treatment volume requirements cannot be solved by applying the same operating conditions. Different steps have to be applied first for nitrogen and then for phosphorus removal recovery.

Initially, longer aerobic phases instead of split aerobic periods with the same sludge residence time (SRT) improve nitrification. A transient response due to alternating conditions has to be avoided to enhance ammonium oxidation. Later, the optimization of the water distribution during a step-feed cycle is required to enhance the denitrification performance. Once nitrification is recovered, an increase in the first feed volume reduces nitrate accumulation in the effluent while denitrification occurs in accordance with the organic matter supplied in the second feed. The reduction of nitrate in the effluent and the availability of organic matter in the first feed improve the EBPR performance and avoid *Competibacter* growth. When nitrogen and phosphorus removal are active again in the system, the optimal configuration can be applied by adjusting the length of some phases to help to raise efficiency and bacterial population growth. Usually, a long enough anaerobic phase to uptake all organic carbon from the wastewater and stabilize phosphate release is required. Anaerobic test to study the phosphate release rate and the hydrolysis of organic matter has to be performed beforehand. For all this reasons, BNR systems have to operate dynamically, and this can easily be achieved using SBR technology.

8.2 Granular formation in an SBR

Granulation has been obtained with short settling times when treating low-strength wastewater, but with loading rates lower than 1 Kg $COD \cdot m^{-3} d^{-1}$.

Applying higher volume exchange ratios (VERs) to obtain higher loading rates, such as 50% or 60% rather than 40%, led to granule instability due to filamentous bulking in this study. Bringing about granulation by increasing the VER induces the presence of more homogenous but smaller particles, causing a major washout of biomass.

Raw wastewater has been proven to be more suitable for granular formation than decanted wastewater because of the higher loads obtained. However, a long start-up is required with low-strength influents (250 days), even when treating raw wastewater or decanted wastewater dosed with ethanol with similar organic loads.

8.3 Biological Nutrient removal in a granular SBR

Nutrient removal is not directly affected by the granulation process, but it is affected by biomass washout during the start-up. Granular sludge cultivated with domestic wastewater was able to treat organic matter with efficiencies of around 86%. Nitrogen and phosphorus removal were unstable during granulation due to poor SRT and mixed liquor suspended solids (MLSSs) concentrations. Once these parameters had been recovered, complete BNR was achieved using synthetic wastewater. The granulation bottleneck for nitrogen and phosphorus removal has proven to be the availability of organic matter for both particles enhancement and nutrient removal, either coming from the hydrolysis of organic particles from raw wastewater or from the addition of an external carbon source. Ethanol used to increase organic loading rates was effective when dealing with poor nutrient removal during granule formation.

Granular sludge allows interactions between different microorganisms and phosphorus accumulating organisms (PAOs) can take advantage of this using both nitrite and nitrate as products of nitrification under aerobic conditions if there is oxygen diffusion in the granules. Both electron acceptors (nitrite and nitrate) have similar rates for phosphate uptake. However, nitrite would enhance simultaneous nitrification denitrification and phosphorus removal (SNDPR) efficiency as it requires less organic matter per unit of phosphate uptake.

8.4 Future perspectives

Granular sludge development presents some issues (e.g. time wasting start-up or difficult to stabilize) when low-strength wastewater, such as domestic, were treated. Regarding to this topic, some points should be further investigated:

- What causes the cyclical dynamics in terms of granule concentration and stability?

- Which is the real starting point for granular development? Are broken granules helping the formation of new granules or disturbing the process?

- Which would be the influence of pre-fermented wastewater in granular development?

- Which is the role of influent characteristics in granulation? Would a wastewater with more industrial composition (e.g. from a 100.000 population equivalent WWTP) have the same behavior in granular development?

- How would be the start-up decreased? Is the granule going to have the same long start-up period in a pilot plant with a higher volume?

- What it would be the scaling-up key parameters?

Furthermore, due to the granule cycle dynamics it has been difficult to properly study the BNR performance. For these reasons, if stable granule could be obtained, some experiments could be carried out:

- How different are the rates for nitrogen and phosphorus removal from a floccular and a granular sludge?

- Is it granular sludge really reducing the carbon source requirement for both denitrification and phosphorus removal or are these processes limited by substrate diffusion?

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Chapter 9. List of references

All the literature used in this thesis is summarized in this chapter.

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ANNEX

Curriculum vitae

Personal information

MARTA COMA BECH

Surname (s):	**COMA BECH**
Name:	**MARTA**
Date of birth:	**18/08/1982**
Address:	**Laboratory of Chemical and Environmental Engineering (LEQUIA-UdG)** **Facultat de Ciències. Campus Montilivi s/n** **17071, Girona (Catalonia, Spain)**
Land line phone:	**0034 972 18 32 49**
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Training received

University Education

Current thesis in the Environmental PhD program for the University of Girona (2007-2011)

Title dissertation: "Biological nutrient removal: from floccular to granular sludge". Supervisors: J. Colprim; S. Puig.

Master thesis in the Environmental Science for the University of Girona (2005-2007)

Title dissertation: "SBR technology: application to biologic treatment of organic matter, nitrogen and phosphorus". Supervisors: J. Colprim; M.D. Balaguer (July, 2007)

Bachelor in Chemical Science for the University of Girona (2000-2005)

Final project dissertation: "Influence of carbon source for biological phosphorus removal from wastewaters". Supervisors: J. Colprim; M.D. Balaguer (September, 2005)

Courses

Laboratory management and Microbiological lab management (PC2 lab) (July 2008)

St Lucia, Brisbane. Organised by University of Queensland.

Confocal Laser Scanning Microscope (CLSM) course for the Universitat Autònoma de Barcelona (Sept 2007)

Barcelona. 26 – 28th September 2007. Organised by Serveis de Microscòpia from UAB.

Laboratory management course for the University of Girona. (Jan 2006)

Girona. 23th January 2006. Organised by Labour Health Office from UdG.

Training Experience

Laboratory of Chemical and Environmental Engineering (LEQUIA-UdG), Institute of the Environment, University of Girona (2006-2010)

Predoctoral Scholarship of the Spanish Government, Ministry of Education and Science, within the FPI program (BES-2006-12277)

Laboratory of Chemical and Environmental Engineering (LEQUIA-UdG), Institute of the Environment, University of Girona (2005-2006)

Collaborating Scholarship with the Laboratory of Chemical and Environmental Engineering (LEQUIA)

Figueres Wastewater Treatment Plant (Jul-Aug 2004)

Colaborating Scholarship for laboratory analysis with FISERSA (Figueres, Spain)

Scientific or technological activity

Participation in R&D&I projects funded by public bodies

Biological nutrient removal (BNR) in GRAnular systems: MEtabolism and Bacterial population (GRAMEB) (2010-2011)
Funding body: Spanish Ministry of Science and Innovation, PT2009-0047

Consolidated groups: Laboratory of Chemical and Environmental Engineering (LEQUIA) (2009-2013)
Funding body: Catalan Goverment, AGAUR, 2009SGR 620.

A new 1-stage advanced GRAnular Sequencing batch reactor (SBR) for biological nutrient Removal (BNR): design and operaTion within an Advanced Control system (GRASTAC). (2009-2011)
Funding body: Spanish Ministry of Education and Science, CTQ2008-06865-C02-01/PPQ

Conception of the Sewage Treatment Plant of the XXI Century. Development, implementation and evaluation of Technologies for the treatment and resources recovery from wastewaters (CONSOLIDER) (2007-2012)
Funding body: Spanish Ministry of Education and Science, CSD2007-00055.

Development of an intelligent control system for a Sequencing Batch Reactor (SBR) for organic matter, nitrogen and phosphorus removal (SICOTIN). (2005-2009)
Funding body: Spanish Ministry of Education and Science,DPI2005-08922-C02-01

Consolidated groups: Laboratory of Chemical and Environmental Engineering (LEQUIA) (2005-2009)
Funding body: Catalan Goverment, AGAUR , 2005SGR 00406.

Participation in R&D&I contracts, agreements or projects with companies and/or governments

Electricity generation from industrial wastewater treatment (2009-2012)
Funding body: Befesa Agua, SAU, 082/09 07/12/09

Residencies in R&D&I centers

REQUIMTE. Universidade Nova de Lisboa (Caparica, Portugal) (2010)
Metabolic and biological analysis from activated sludge. Supervision: Prof. M.A.M. Reis, Dr A. Oehmen, Dr G. Carvalho. May- August 2010.

Advanced water management centre (AWMC), University of Queensland, St Lucia (Brisbane, Australia) (2008)
Aerobic granular treatment of domestic wastewater. Supervision: Prof. Z. Yuan, Dr P. Bond, Dr M. Pijuan. July-December 2008.

Assistance to conferences

1st IWA Spain National Young Water Professional Conference. Barcelona (Spain), 16-18th June 2010. Organized by IWA.

IWA Conference on Sustainable Solutions for Small Water and Wastewater Systems (S2SMALL). Girona (Spain) 19-22nd April 2010. Organized by LEQUIA and IWA.

2nd IWA Specialized Conference on Nutrient Management in Wastewater Treatment Processes. Krakow (Poland), 6-9th September 2009. Organized by IWA.

EBCRC annual conference. Adelaide (Australia), 8-10 December 2008. Organized by EBCRC Australia.

AWMC Showcase and Networking Event. Brisbane (Australia), 28 October 2008. Organized by AWMC (The University of Queensland).

4th Sequencing Batch Reactor Technology Conference. Roma (Italy), 7-10 April 2008. Organized by IWA.

CLONIC Final Workshop. Barcelona, 19 – 20 Abril 2007. Organized by CESPA and LEQUIA-UdG.

ANAMMOX Workshop. **State of the art.** Torre AGBAR, Barcelona. April 18th, 2007. Organized by LEQUIA-UdG.

Book chapters

1. Puig, S., Serra, M., Cabré, M., Coma, M., Balaguer M.D., and Colprim, J. (2010)
   "Wastewater treatment and electricity production by microbial fuel cells (MFCs)"
   Integral water cycle: present and future, Ed: ANQUE (Oviedo (Spain)). ISBN:978-84-693-2258-1.

Journal papers

1. Puig, S., Serra, M., Coma, M., Balaguer, M.D. and Colprim, J. (2011) "Simultaneous domestic wastewater treatment and renewable energy production using microbial fuel cells (MFCs)" *Water Sci Technol* (accepted) ISSN: 0273-1223.

2. Puig, S., Serra, M., Coma, M., Balaguer, M.D. and Colprim, J. (2011) "Microbial fuel cell application in landfill leachate treatment" *J. Hazard. Mater*., 185 (2-3), 763-767.

3. Coma, M., Puig, S., Balaguer, M.D., Colprim, J. (2010) "The role of nitrate and nitrite in a granular sludge process treating low-strength wastewater". *Chem Eng J*., 164 (1), 208-213.

4. Puig, S., Serra, M., Coma M., Cabré, M., Balaguer, M.D and Colprim, J. (2010) *"Effect of pH on nutrient dynamics and electricity production using microbial fuel cells" Bioresource Technol*., 101, 9594-9599.

5. Coma, M., Puig, S., Monclús, H., Balaguer M.D. and Colprim J.(2010) *"Effect of cycle changes on simultaneous biological nutrient removal in an SBR" Environ Technol*., 31 (3), 285-294.

6. Monclús, H., Puig S., Coma, M., Bosch, A., Balaguer, M. D. and Colprim, J. (2009) *"Nitrogen removal treating landfill leachate using the SBR technology" Environ Technol*., 30 (3), 283-290.

7. Puig, S., Coma, M., Monclús, M., van Loosdrecht, MCM., Colprim, J. and Balaguer, MD. (2008) *"Selection between alcohols and volatile fatty acids as external carbon sources for EBPR" Water Res*., 42(3), 557-566.

8. Puig, S., Coma, M., van Loosdrecht, MCM., Colprim, J. and Balaguer, MD. (2007) *"Biological nutrient removal in a sequencing batch reactor using ethanol as the carbon source" J Chem Technol Biot*., 82(10), 898-904.

Conference proceedings

1. Wong Ramírez, A., Colomer Llinàs, J., Coma, M., Colprim, J. (2010) *"PCA Intelligent Contribution Analysis for Fault Diagnosis in a Sequencing Batch Reactor" International Congress on Environmental Modelling and Software* , (5-8 July, Ottawa, Ontario, Canada), *Oral*.

2. Verawaty, M.; Coma, M.; Yuan, Z.; Pijuan, M.; Bond, P.L. (2010) *"Application of the aerobic granular technology to treat domestic wastewater for biological nutrient removal" Australia's National Water Conference and Exhibition*, (8-10 March, Brisbane, Australia), *Oral*.

3. Puig, S., Serra, M., Cabré, M., Coma, M., Balaguer M.D., and Colprim, J. (2010) *"Wastewater treatment and electricity production by microbial fuel cells (MFCs)" 7th ANQUE's International congress integral water cycle: present and future*, (13th-16th June, Oviedo, Spain), *Oral*.

4. Coma, M., Puig, S., Balaguer M.D., and Colprim, J. (2010) *"Challenges of Granulation on Biological Nutrient Removal Systems" 1st IWA Spain National Young Water Professionals Conference*, (16th-18th June, Barcelona, Spain), *Oral*.

5. Puig, S., Serra, M., Coma, M., Balaguer, MD. and Colprim, J. (2010) *"Simultaneous domestic wastewater treatment and renewable energy production by microbial fuel cells (MFCs)" IWA Conference on Sustainable Solutions for Small Water and Wastewater Systems*, (19-22 April, Girona, Spain), *Oral*.

6. Coma, M., Puig, S., Barceló, M., Balaguer, M.D. and Colprim, J.(2010) *"Influence of primary treatment on nutrient removal from domestic wastewater: moving to granular sludge" Sustainable Solutions for Small Water and wastewater Treatment systems (S2Small2010)*, (19-22 April, Girona, Spain), *Oral*.

7. M. Coma, S. Puig, N. Serón, M.D. Balaguer and J. Colprim (2009) *"Granular sludge development at different exchange ratios for nutrient removal" 2nd IWA specialized conference on nutrient management in wastewater treatment processes*, (6-9 September, Krakow, Poland), *Oral*.

8. H. Monclús, S. Puig, M. Coma, MD. Balaguer, J. Colprim (2008) *"Treatment of high N loaded leachates using the SBR technology: Practical experiences" 4th Sequencing Batch Reactor (SBR) Technology Conference*, (7-10 April, Rome, Italy), *Poster*.

9. Coma, M., Puig, S., Monclús, H., Balaguer, MD. and Colprim, J. (2008) *"Sludge granulation in an SBR for phosphorus removal" 4th Sequencing Batch Reactor Technology*, (7-10 April, Rome, Italy), *Poster*.

10. Puig, S., Coma, M., Monclús, H., van Loosdrecht, MCM., Colprim, J. and Balaguer, MD. (2008) *"Ethanol as a carbon source for biological nutrient removal from wastewaters" 4th Sequencing Batch Reactor Technology* , (7-10 April, Roma, Italy), *Oral*.

11. Balaguer, M.D., Puig, S., Corominas, Ll., Coma, M., and Colprim, J. (2006) ***"****Eliminación de material orgánica,nitrógeno y fósforo mediante un reactor secuencial por cargas (SBR): operación y control" Mesa Española de tratamiento de Aguas* , (13-14 March, Valencia, Spain), *Oral*.

In preparation

Coma, M., Pijuan, M., Verawaty, M., Colprim, J., Bond, P., Yuan, Z. "Granulation for biological nutrient removal treating domestic wastewater". (Journal, in preparation).

Coma, M., Puig, S., Oehmen, A., Carvalho, G., Balaguer, M.D., Colprim, J. "Influence of matrix wastewater in granulation for sewage treatment". (Journal, in preparation).

Batchellí, L., Puig, S., Serra, M., Coma, M., Cabré, M., Balaguer, MD., Colprim, J. "Domestic wastewater treatment using MFCs: nutrient removal and pH effect". (Spanish Young Water Conference, 15th-17th June 2011, Madrid, Spain).

Collection of other credits

1. **Description credits:** Science diffusion: Research night 2009 (Girona)
   Body conferring the credit and date: University of Girona (25/09/2009)

2. **Description credits:** Science diffusion: "Practical work in a research group" (show to High School students how a research group works).
   Body conferring the credit and date: University of Girona (06/07/2009)

3. **Description credits:** Courses and seminars- Given: Fluorescence in situ Hybridization (FISH) course (internal course)
   Body conferring the credit and date: Laboratory of Chemical and Environmental Engineering (25/03/2009)

4. **Description credits:** Science diffusion: Education showcase (Barcelona, 2009)
   Body conferring the credit and date: University of Girona (18/03/2009)

5. **Description credits: Reviewer:** Letters in Applied Microbiology (LAM)
   Body conferring the credit and date: Willey-Blackwell (2009)

6. **Description credits: Reviewer:** Chemical Engineering Journal (CEJ)
   Body conferring the credit and date: Elsevier (2010)

7. **Description credits: Reviewer:** 2nd IWA Spain National Young Water Professionals Conference
   Body conferring the credit and date: International Water Association (2010-2011)

8. **Description credits: Reviewer:** Biotechnology and Bioengineering
   Body conferring the credit: Wiley (2011)

9. **Description credits: Reviewer:** Environmental Technology
   Body conferring the credit and date: Elsevier (2011),